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Original Article

In vitro release and anti-herpetic activity of *Cymbopogon citratus* volatile oil-loaded nanogel

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ABSTRACT

This study aimed to prepare hydrogel containing *Cymbopogon citratus* (DC.) Stapf, Poaceae, volatile oil encapsulated in poly (D,L-lactide-co-glycolide) nanoparticles and to evaluate its *in vitro* anti-herpetic activity. Polymeric nanoparticles were prepared by solvent emulsification-diffusion method and incorporated in carbomer hydrogels. *In vitro* release profiles for the nanogel, loaded nanoparticles and hydrogel containing free oil were evaluated by dialysis. Inhibitory activities against Herpes simplex for the formulations were investigated in Vero cells. Hydrogel was developed using nanoparticles with mean diameter of 217.1 nm and negative Zeta potential (−20.5 mV). Volatile oil release profile showed a biphasic pattern with an initial faster release and subsequent sustained phase in all formulations. Nanogel strongly inhibited virus in a non-cytotoxic concentration, 42.16 times lower than free oil, 8.76 and 2.23 times than loaded nanoparticles and hydrogel containing free oil, respectively. These results highlight the potential of nanogel to protect oil against volatilization, control release and improve its anti-herpetic activity.

Introduction

Genital and oral infection caused by Herpes simplex virus (HSV), serotypes 1 (HSV-1) and 2 (HSV-2) are highly prevalent around the world (Bernstein et al., 2013), and serious systemic illnesses can be caused in immunocompromised patients and neonates. HSV has the ability to infect and replicate in mucocutaneous cells and migrate to the sensory neurons, establishing latency after incubation. The latent virus is reactivated spontaneously causing clinical recurrences characterized by herpetic lesions in the primary sites of infection (Kahan et al., 2005; Fatahzadeh and Schwartz, 2007).

Monotherapy with nucleoside analogs, such as acyclovir, valacyclovir or famciclovir, is commonly used to shorten the course and decrease the severity of these HSV-related clinical symptoms (Kahan et al., 2005; Astani et al., 2011). However, the increase of drug resistance and cytotoxicity, as well as the high cost of systemic long-term therapy enhance the need for new effective therapeutic compounds against viral infections (Kahan et al., 2005; Astani et al., 2011).

Medicinal plants produce a wide variety of chemical constituents which are of great interest as potential topical microbicides for inhibiting viral replication, and consequently for controlling viral infection. Furthermore, easy administration reduced systemic exposure to bioactive compounds and low cost are major benefits of topical antiviral agents from natural sources, especially in patients with frequent clinical recurrences (Kahan et al., 2005; Thompson, 2006; Astani et al., 2011).

Cymbopogon citratus (DC.) Stapf is a perennial herb originated from India, which belongs to Poaceae family, and is widely cultivated in the tropics and sub-tropics. Its volatile oil (VO), also known as lemongrass, is obtained from fresh leaves and used in food, cosmetics and pharmaceutical industries (Negrelle and Gomes, 2007). Its major compound, citral, a natural mixture of the isomers geranial (α-citral) and neral (β-citral) (Negrelle and Gomes, 2007) characterize this oil. Bioactivity studies have demonstrated that *C. citratus* volatile oil (CcVO) exhibits biological activities, such as antibacterial (Pereira et al., 2004; Oloyede, 2009; Naik et al., 2010; Aiemsaard et al., 2011), antioxidant (Pereira et al., 2009), antifungal (Silva et al., 2009), antinociceptive peripheral and central (Viana

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et al., 2000), sedative (Pereira et al., 2004) and anti-herpetic against HSV-1 (Minami et al., 2003).

The use of VO in topic pharmaceutical products shows limitations due to its high volatility and instability. Thus, nanoencapsulation and subsequent incorporation into semisolid formulations offer an interesting alternative to modulate its permeation, to improve its distribution on the surface of the skin, and to confer protection against degradation and volatilization (Bouchemal et al., 2004; Guterres et al., 2007; Förster et al., 2009; Falcão et al., 2015).

Flores et al. (2013) demonstrated that poly (ε-caprolactone) (PCL) polymeric nanocapsules containing *Melaleuca alternifolia* VO have ability to reduce *Trichophyton rubrum* growth in a nail infection model. Abreu et al. (2012) developed a chitosan/cashew gum nanogel containing *Lippia sidoides* VO. This formulation provided VO sustained release and showed a *St. Aegypti* larvae mortality higher than free oil. Recently, our group reported CcVO encapsulation in PCL polymeric nanoparticles and its molecular complexation in β-cyclodextrin, showing how promising PCL matrix is for encapsulating the volatile oil (Falcão et al., 2011).

Based on the considerations above, the goal of this study was to prepare nanoparticulate semisolid topical hydrogel to enhance CcVO anti-herpetic activity. For this purpose, the aims of the present study were to formulate a hydrophilic gel incorporated with CcVO encapsulated in polymeric nanoparticles and to evaluate its *in vitro* anti-herpetic activity. Polymeric nanoparticles were produced by solvent emulsification-diffusion method, using poly (D,L-lactide-co-glycolide) (PLGA) as wall material, and poly (vinyl alcohol) (PVA) as stabilizer. After size distribution, Zeta potential, and encapsulation efficiency characterization, nanoparticles were incorporated in Carbopol® Ultrez hydrophilic gel. The citral content was measured to estimate the active stability. The *in vitro* release profile of the volatile oil from nanogel was investigated using dialysis technique. Inhibitory activities against Herpes simplex types 1 and 2 were evaluated in Vero cells by the titer reduction assay and compared with free CcVO, loaded nanoparticles and hydrogel containing free oil.

Materials and methods

Plant material

Cymbopogon citratus (DC.) Stapf, Poaceae, fresh leaves were collected at Laboratory of Cultivation and Biomass Production of Farmaguinhos/Fiocruz- Jacarepaguá campus, Rio de Janeiro, Brazil, during August 2012. A voucher specimen was deposited at Rio de Janeiro Botanical Garden Herbarium Herbarium under the number RB3273021.

Chemicals, cells and viruses

Poly (vinyl alcohol) (PVA; Mw 85,000–124,000 Da), poly (D,L-lactide-co-glycolide) (PLGA; Mw 50,000–75,000 Da) with a lactide:glycolide molar ratio of 85:15 and citral standard reference were purchased from Sigma–Aldrich (São Paulo, Brazil). Carbopol® Ultrez 10 NF was supplied from Fagron (São Paulo, Brazil). Ethyl acetate and triethanolamine from Vetec (Rio de Janeiro, Brazil). UV/HPLC grade dimethyl sulfoxide (DMSO) was acquired from Tedia (São Paulo, Brazil). Phosphate-buffered saline (PBS) pH 6.8 was prepared as described by United States Pharmacopeia (2008), using sodium phosphate dibasic anhydrous, sodium phosphate monobasic anhydrous and sodium dodecyl sulfate (SDS) provided by Vetec (Rio de Janeiro, Brazil). All other chemical reagents were commercial products of analytical or reagent grade and were used without further purification.

Vero cells (African green monkey kidney cells; Rio de Janeiro Cell Bank, Brazil) were grown in Eagle's minimum essential medium (MEM; Cultilab, Campinas, Brazil) supplemented with 2 mM L-glutamine (Sigma–Aldrich, São Paulo, Brazil), 50 μg/ml garamicin, 2.5 μg/ml fungizon (Gibco, Gainthersburg, USA), 0.25 mM of sodium bicarbonate solution (Merck), 10 mM of 4-(2-hidroxietil)-1-piperazineethanesulfonic acid (HEPES) (Sigma–Aldrich, São Paulo, Brazil), plus 10% of heat-inactivated fetal bovine serum (FBS; Cultilab, Campinas, Brazil) and maintained at 37 °C in atmosphere of 5% of CO_2. Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) were isolated from diagnosed patients in the Virology Department of the Federal University of Rio de Janeiro, Brazil. Viruses were typed by polymerase chain reaction (PCR) using specific primers for identification (Markoulatos et al., 2001).

Extraction of volatile oil

Cymbopogon citratus VO was extracted, right after leaves were collected, by hydrodistillation using "Clevenger type apparatus" from fresh leaves (2.5 kg), which were cut in small pieces. This process was performed for 1 h after the solution started boiling. Hydrolate was collected and centrifuged at 4800 × *g* force for 5 min (Anvisa, 2010). The VO was recovered in an amber glass bottle and stored under refrigeration. The yields were calculated according to the weight of the plant material before distillation (expressed in percent, w/w of the dry plant material).

Qualitative and quantitative analysis of the volatile oil

Qualitative analysis of the CcVO volatile components was performed by gas chromatography–mass spectrometry (GC–MS) with a 6890 N (Agilent Technologies, USA) equipped with a mass detector 5973 Network (Agilent Technologies, USA), an injector 7683B Series (Agilent Technologies, USA) and a DB-5MS column (30 m × 0.32 mm, 0.25 μm film thickness). Mass ranged from 40 to 600 *m/z* (GC–MS). Helium gas was used as carrier at a flow rate of 0.5 ml/min. The oven temperature program was 40 °C, increasing 4 °C/min until it reached 290 °C, with a 5-min isotherm. The volume 1 μl of samples was injected. The components were identified via peak matching with Wiley 7 N mass spectra library. Homologous series of *n*-alkanes (C7-C26; C28; C30) were used as reference points in the calculation of retention index, which were compared with the literature (Adams, 2007).

High-performance liquid chromatography (HPLC) analyses were performed with a Shimadzu chromatography, equipped with a CTO-20AC photodiode array detector and LC-20AT pump, using XBrigdeTM C18 column (4.6 mm × 150 mm, 50 μm) connected by a C18 precolumn Shim-pack GVP-ODS (10 × 4.6 mm). The mobile phase consisted of water (solvent A) and acetonitrile (solvent B) sonicated with 0.05% trifluoroacetic acid at a constant flow rate of 0.8 ml/min, mixed in linear gradients as follows: $t = 0$ A:B (40:60, v/v), reaching 100% B in 28 min and finally at A:B (40:60, v/v) until 40 min. The injection volume was 90 μl. Quantification of citral was carried out (λ = 239 nm) by measuring the peak areas in relation to the citral standard reference solubilized in acetonitrile HPLC grade. A calibration curve in the range 0.25–20 μg/ml was constructed by means of the least-square method ($r = 0.9980$, $y = 3566.2x + 664{,}678$).

Preparation of nanoparticles

Cymbopogon citratus VO-loaded nanoparticles (NPVO) were prepared by the solvent emulsification-diffusion method (Moinard-Chécot et al., 2008) with adaptations. First, mutually saturated aqueous and organic phases were prepared. PLGA (350 mg) was dissolved in 10 ml of ethyl acetate previously saturated with

distilled water, and then CcVO (350 mg) was added (organic phase). Meanwhile, PVA (1%, w/v) was solubilized in 40 ml of distilled water previously saturated with ethyl acetate (aqueous phase). The organic phase was added dropwise into the aqueous phase in an ice cool bath, under continuous emulsification using ultrasonic homogenizer (Sonic Ruptor 250, Omni International, USA) at 100 w for 5 min. The oil-in-water emulsion formed was diluted with distilled water under magnetic stirring in order to allow the solvent diffusion. The resulting nanosuspension was frozen, lyophilized and stored at 4 °C in an airtight bottle. Unloaded PLGA nanoparticles (NP) were used as reference. Experiments formulations were carried out in triplicate.

Size distribution and Zeta potential of the nanoparticles

Nanoparticles were characterized according to their size distribution and Zeta potential by dynamic light scattering (DLS) and electrophoretic mobility, respectively, using Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK). Samples were resuspended in Milli-Q water (1:25) and measurements were performed in triplicate, at 25 °C, using water refractive index (1.330). The results were expressed as the mean ± standard deviation (SD) (Fernandes et al., 2013).

Initial drug loading and entrapment efficiency (EE %)

The total content of encapsulated oil in PLGA nanoparticles was determined by the solvent extraction method (Falcão et al., 2011). Lyophilized nanoparticles were solubilized with DMSO, vigorously mixed with Vortex® apparatus, and centrifugated at 2000 × *g* force for 1 min. The supernatant was immediately analyzed by UV-Vis spectrophotometry (Shimadzu® UV-2600, Japan) at 260 nm (the maximum absorption wavelength for CcVO in DMSO). Unloaded nanoparticles were used as a reference. CcVO content was calculated from an average of three standard curves in the range 0.001–0.08 mg/ml ($r = 0.9993$, $y = 26.144x + 0.0409$). *C. citratus* VO loading (mg CcVO/g NP) and entrapment efficiency (EE) were calculated as shown in Eqs. (1) and (2), respectively. The extraction procedures and analyses were carried out in triplicate.

$$\text{VO loading} = 100 \times (\text{weight of VO in particles/weight of particles}) \quad (1)$$

$$\text{EE (\%)} = 100 \times \left(\frac{\text{measured CcVO content}}{\text{theoretical total CcVO content}}\right) \quad (2)$$

Hydrogel preparation

Briefly, Carbopol® Ultrez (0.5% w/w) was dispersed in distilled water (88.1% w/w) under constant magnetic stirring until complete dispersion. *C. citratus* VO loaded nanoparticles, unloaded nanoparticles or CcVO (11.4% w/w) were incorporated under gentle homogenization and subsequently neutralized with triethanolamine (pH 6–7). Hydrogel blank (control) was prepared with the same method but no oil or nanoparticle was added. Formulations are referred herein as: hydrogel containing CcVO-loaded nanoparticles (HNPVO); unloaded nanoparticles (HNP); free CcVO (HVO) and hydrogel blank (HB). All formulations were prepared in triplicate. A sample of each gel was packaged in a glass bottle with screw cap and bung sealing, and kept at 4 °C. CcVO and citral content in the hydrogels were monitored by HPLC after 60 days of storage.

In vitro *CcVO release*

In vitro release of CcVO from NPVO, HNPVO and HVO was investigated using dialysis membranes, according to Jeong et al. (2008), with adaptations. NP, HNP and HB were used as controls. The system consists of (1) a donor compartment covered by a dialysis membrane (Sigma–Aldrich, Mw cutoffs: 12,000 Da), where the sample was added, and (2) a receptor compartment containing PBS with SDS (1%, w/v), pH 6.8 (USP 31), maintained at 37 °C ± 0.5 under magnetic stirring (100 rpm). At pre-determined time intervals (5, 10, 15, 30, 60 min, and then, every hour until 24 h) samples were withdrawn (replaced with fresh medium) and immediately analyzed by UV-Vis spectrophotometry at 240 nm (wavelength of maximum absorption for CcVO in PBS/SDS medium). CcVO content was calculated from an average of three standard curves prepared with dissolution media, ranging from 0.00025 to 0.08 mg/ml ($r = 0.9999$, $y = 71.767x + 0.0144$). Each sample was evaluated in triplicate during 24 h.

In vitro release curves were subsequently linearized to obtain the oil dissolution efficiency (DE). Furthermore, the mathematical models of zero order (3), first order (4), Higuchi (5), Hixson–Crowell (6) and Korsmeyer–Peppas (7) were applied to calculate the volatile oil kinetics of release and to elucidate the release mechanism.

$$Q = k_t + Q_0 \quad (3)$$

$$Q = Q_0 \cdot e^{kt} \quad (4)$$

$$Q = k \cdot t^{0.5} \quad (5)$$

$$Q^{1/3} = k \cdot t + Q_0^{1/3} \quad (6)$$

$$Q = k \cdot t \quad (7)$$

Q represents the cumulative amount of active released during time t, Q_0 the initial loading of the oil in the formulation, k a release constant, and n the release exponent indicative of the CcVO release mechanism.

Cytotoxicity assay

Cymbopogon citratus VO, HVO, HB, NPVO, HNPVO and NP were solubilized in water (400 μg/ml) and the free VO was solubilized in DMSO (1% v/v). Solutions were sterilized by filtration through a Millipore membrane (0.22 μm) and frozen at −20 °C until use. The cytotoxicity assay was performed prior to antiviral tests by incubating Vero cell monolayers (96-well microplates) with two-fold serial dilutions (1.9–250 μg/ml) of the test samples, for 48 h at 37 °C, in a 5% CO_2 atmosphere. Morphological alterations of the treated cells were observed under an inverted optical microscope and the maximum nontoxic concentrations (MNTC) were determined (Rodriguez et al., 1990). Cellular viability was evaluated by the neutral red dye-uptake method (Borenfreund and Puerner, 1985). The 50% cytotoxic concentration (CC_{50}) was defined as the one that was able to cause a 50% reduction in the number of viable cells.

Antiviral activity assay

The antiviral activities of the samples were measured by HSV-1 and HSV-2 titer reduction. Virus titers were calculated using Reed and Müench statistical method (Reed and Müench, 1938) and expressed as 50% tissue culture infective dose ($TCID_{50}$) per ml.

Vero cell monolayers were treated with samples at the MNTC and 100 $TCID_{50}$/ml. HSV-1 or HSV-2 suspensions were added to treated and untreated cell cultures and incubated at 37 °C for 48 h, in a 5% CO_2 atmosphere. After incubation, the supernatant was collected and virus titers were measured. The antiviral

Table 1
Chemical composition of main constituents of lemongrass (*Cymbopogon citratus*) by GC–MS analysis.

Compounds	Retention Index (RI)[a]	Relative content (%)
Camphene	953	0.29
6-Methyl-5-hepten-2-one	988	0.19
Limonene	1032	0.99
Linalool	1100	0.42
cis-Crisantenol	1164	0.76
n-Decanal	1207	0.19
Neral	1243	36.37
Geraniol	1253	2.66
Geranial	1273	53.20
2-Undecanone	1293	0.22
Geranyl acetate	1379	1.50
trans-Caryophyllene	1423	1.03
γ-Cadinene	1516	0.27
Caryophyllene oxide	1587	0.59
Identified compounds	–	98.39

[a] Retention index (RI) was calculated and compared with the literature (Adams, 2007).

activity was expressed as percentage inhibition (PI) (Nishimura et al., 1977), using antilogarithmic $TCID_{50}$ values as follows: PI = [1 (antilogarithmic test value/antilogarithmic control value)] × 100. The dose–response curve was established starting from MNTC, and the 50% effective concentration (EC_{50}) was defined as the concentration required for 50% protection against virus-induced cytopathic effects. The selectivity index (SI) was determined as the ratio of CC_{50} to EC_{50}. The experiment was performed in triplicate and repeated three times.

Statistical analysis

All analyzes were performed in triplicate and results expressed as mean ± SD (standard deviation). Results were analyzed using Student 's *t*-test or one-way ANOVA to assess the significance of the differences among data employing GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, USA). The criterion for statistical significance was $p < 0.05$.

Results and discussion

Cymbopogon citratus volatile oil

Fresh and fragmented leaves were subjected to hydrodistillation, and a yellow colored oil with characteristic odor of lemon was obtained, yielding 0.37% (w/w). This result is within the range previously described of 0.28 to 1.4% (Negrelle and Gomes, 2007).

Cymbopogon citratus VO qualitative analysis by GC–MS allowed the identification of fourteen chemical components. Retention index (RI) and the percentage compositions are shown in Table 1.

The major component, citral (89.57% of the chromatogram relative area), was composed by geometric isomers neral (36.37%) and geranial (53.20%), in agreement with the quality parameter for this volatile oil (Schaneberg and Khan, 2002). Other isolated components, such as geraniol, geranyl acetate and *trans*-caryophyllene were found higher than 1% in total oil.

Microclimatic and phytogeographic factors, geographical and agronomical conditions as well as genotype plants can affect the quantity and chemical composition of volatile oils (Tajidin et al., 2012). In a previous work, Pinto et al. (2015) showed the same citral content in *C. citratus* cultivated at same location and under similar conditions. Falcão and collaborators (2011) also obtained CcVO from the same geographic origin but, as well as in the present work, authors did not find myrcene in its composition, despite this monoterpene is usually reported (Negrelle and Gomes, 2007).

Obtaining of the nanoparticles

Cymbopogon citratus VO was encapsulated in PLGA matrices by the solvent emulsification-diffusion technique. PVA was used as a stabilizing agent, what facilitates the formation of small easily-redispersible particles in aqueous medium (Sahoo et al., 2002). Table 2 summarizes the results on the physicochemical characterization of nanoparticles.

Nanoencapsulation forms from CcVO have been developed by our group in order to improve its stability and control release. In our previous work (Falcão et al., 2011), nanoparticles formulated with PCL incorporated more compounds and exhibited smaller mean diameter (240.0 ± 3.4 nm) than CcVO/β-cyclodextrin complex (441.2 ± 14.0 nm). Therefore, CcVO encapsulation in PCL biodegradable nanoparticles was more effective when compared to molecular inclusion in β-cyclodextrin. Unpublished study conducted by our group showed that PLGA nanoparticles containing CcVO have higher encapsulation efficiency and better release profile *in vitro* than PCL nanoparticles. For this reason, PCL was substituted by PLGA as wall material aiming to encapsulate CcVO in the current work.

Particles with an average hydrodynamic diameter smaller than 250 nm and unimodal distribution profile, considered moderately homogeneous (PDI < 0.500), were obtained (diameters smaller than 600 nm are recommended for pharmaceutical and cosmetic formulations for topical application; Bouchemal et al., 2004). *C. citratus* VO addition in NP did not change the mean diameter ($p > 0.05$, Student's *t*), with a result consistent with a former study with PCL nanoparticles (NP 291.0 nm ± 2.3; NPVO 240.0 nm ± 3.4) (Falcão et al., 2011).

Zeta potential values lower than −10 mV are associated with good physical and chemical stability due to the high repulsion between particles, preventing their aggregation (Mora-Huertas et al., 2010). Both PVA and PLGA created a negative surface charge on the particles (Sahana et al., 2008), consistent with results obtained in colloidal suspensions of NP (−17.8 mV ± 3.8) and of NPVO (−20.5 mV ± 8.8), considered highly stable in dispersion medium (Wu et al., 2011; Clogston and Patri, 2011). Furthermore, it is known that the presence of oil phase in the nanocapsules formation makes absolute values of Zeta potential (−41.94 mV) larger than those presented by nanospheres (−16.33 mV) (Schaffazick and Guterres, 2003), suggesting the possibility of obtaining nanospheres in this study. Deeper analyses would be required to support such a claim, though.

Regarding the content of encapsulated volatile oil, a concentration of 58.59 mg/g ± 0.85 was found in the developed polymeric nanoparticles, with an encapsulation efficiency of 28.48% ± 0.40,

Table 2
Values distribution of particle size, polydispersity index (PI), Zeta potential and encapsulation efficiency (EE) of the PLGA nanoparticles obtained by solvent emulsification–diffusion technique.

Nanoparticles	Mean diameter (nm)	PDI	Zeta potencial (mV)	EE (%)
NP	248.1 ± 27.8	0.480 ± 0.047	−17.8 ± 3.8	–
NPVO	217.1 ± 19.9	0.481 ± 0.023	−20.5 ± 8.8	28.48 ± 0.4

Mean ± standard deviation ($n = 3$); NP, nanoparticles blank; NPVO, nanoparticles containing volatile oil; EE, encapsulation efficiency.

Fig. 1. Hydrophilic gels prepared with Carbopol® Ultrez 10 NF as gelling agent: (1) hydrogel with blank nanoparticles (HNP); (2) hydrogel with CcVO encapsulated in PLGA nanoparticles (HNPVO); (3) hydrogel blank (HB); (4) hydrogel prepared with free CcVO (HVO).

similar to results found when the solvent emulsification-diffusion technique was employed (Cohen-Sela et al., 2009; Zhang et al., 2011).

Cohen-Sela et al. (2009) showed that polymer type and its molecular weight directly affect lipophilic drug encapsulation efficiency. PLGA polymers with a high ratio of lactate and glycolate, and poly (lactic acid) low molecular weight lead to a reduced drug encapsulation rate. This can be related to the low CcVO content since PLGA molar ratio of lactate:glycolate was 85:15. Additionally, Sahana et al. (2008) suggested ethyl acetate as organic phase presented moderate encapsulation efficiency. Despite the high hydrosolubility, ethyl acetate has reduced vapor pressure, slowing PLGA precipitation by splitting drug in the aqueous phase. Furthermore, CcVO low solubility in PVA external phase may result in its loss during particles preparation.

Preparation of hydrogels

Hydrophilic gels employing Carbopol® Ultrez 10 NF as gelling agent were prepared with free CcVO (HVO) and encapsulated in PLGA nanoparticles (HNPVO). Hydrogel blank (HB) and blank nanoparticles (HNP) were also prepared as controls (Fig. 1). Carbomers such as Carbopol® are commonly employed in pharmaceutical preparations. Carbopol® Ultrez 10 NF was chosen for this work due its easiness to disperse in water, avoiding heating and vigorous stirring.

HNPVO seemed macroscopically homogenous, milky, lightly yellow, and opalescent, while HVO was translucent. HNP was whitish, opaque and homogeneous, and HB was colorless. HVO and HNPVO formulations showed characteristic odor of lemon grass oil, though the latter was less pronounced. Thus, polymeric nanoparticles presented the ability to mask physicochemical properties of encapsulated substances (Guterres et al., 2007), making the gel more attractive for reducing the intense CcVO odor.

Initial CcVO content decreased significantly ($p < 0.05$, Student's t) in HVO (18.12% ± 3.8) and HNPVO (17.39% ± 3.3) after hydrogels preparation, similar in both samples. This might occur as a result of the time required for carbomer dispersion in water, resulting in a CcVO loss by evaporation. In contrast, the citral content remained constant after 60 days in both formulations, showing no significant difference between days ($p > 0.05$, ANOVA). Weisheimer et al. (2010) developed microparticles containing CcVO through the precipitation method, using β-cyclodextrin as encapsulant material, and incorporated them into semisolid formulation (non ionic emulsion). A reduction of 13.3% in citral content was found, when compared to free microparticles (40% reduction), showing the stability improvement from the volatile oil (40 °C/110 days). This corroborates with data obtained in the current work, demonstrating greater protection against oil volatilization and degradation through its encapsulation, or simply incorporating it into semisolid bases.

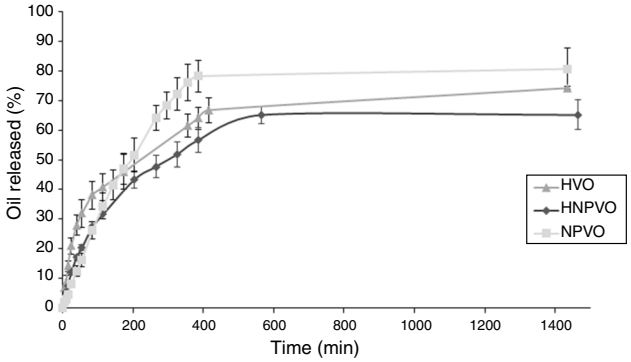

Fig. 2. *Cymbopogon citratus* volatile oil *in vitro* release kinetics encapsulated in PLGA nanoparticles (NPVO), incorporated in its free form in hydrogel (HVO), and incorporated into the same formulation with previous nanoencapsulation (HNPVO) (saline–phosphate buffer with sodium lauryl sulfate (1%), pH 6.8, 37 °C, 100 rpm, $n = 3$).

In view of the obtained results, it is suggested, in the developed hydrogels, the matrix formed by the thickening agent may play a role in protecting the CcVO from volatilization regardless of nanoencapsulation.

In vitro *CcVO release studies*

Fig. 2 shows the CcVO *in vitro* release profiles from NPVO, HNPVO and HVO, up to 24 h. All samples showed a pattern of biphasic release, with a faster release in the initial phase followed by a sustained release at a constant rate, similar to that found in polymeric systems containing natural substances (Gomes et al., 2011; Iannitelli et al., 2011). This rapid initial release can be attributed to the immediate dissolution of the volatile oil fraction adsorbed on the particle surface or next to it, favoring its release. In the second stage, the oil is spread throughout the polymer matrix, and the release rate decreases as a function of time. This is due to oil location in the particle core, reaching more slowly the surface, and consequently taking longer to be released (Mu and Feng, 2003; Gomes et al., 2011).

Table 3
Kinetic behavior of *in vitro* release of the *Cymbopogon citratus* volatile oil from developed formulations.

Sample	Mathematical model										
	Zero Order		First Order		Higuchi		Hixson-Crowell		Korsmeyer-Peppas		
	k_0	R^2	k_1	R^2	k_H	R^2	k_{HC}	R^2	k_K	R^2	n
NPVO	0.209	0.979	0.008	0.760	47.1	0.992	0.005	0.999	0.46	0.995	0.884
HVO	0.127	0.883	0.004	0.638	314.4	0.973	0.003	0.936	35.22	0.961	0.505
HNPVO	0.114	0.904	0.004	0.633	300.3	0.992	0.002	0.949	12.82	0.977	0.653

R^2, coefficient of determination; k, release rate constant; n, diffusion exponent.

Table 4
Cytotoxicity and antiviral activity [values expressed in *Cymbopogon citratus* volatile oil concentration].

Samples	CC_{50} (μg/ml)	MNTC (μg/ml)	HSV-1		HSV-2	
			EC_{50} (μg/ml)	SI	EC_{50} (μg/ml)	SI
CcVO	115.89	31.2	11.59	9.99	6.69	17.32
HNPVO	>1.48	0.74	0.32	>4.67	0.33	>4.50
NPVO	>12.97	6.48	1.32	>9.83	1.34	>9.67
NP	0	0	–	–	–	–
HNP	0	0	–	–	–	–
HB	0	0	–	–	–	–
HVO	>1.65	1.65	–	–	0.71	>2.31

CC_{50}, cytotoxic concentration of volatile oil which reduced viable cell number by 50%; MNTC, maximum non-toxic concentration of volatile oil; EC_{50}, effective concentration of oil that reduced the viral titer by 50%; SI, selectivity index; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; –, not activity observed.

The incorporation of nanoparticles in hydrogel limits the CcVO mobility in the gel polymeric network, leading to a slower diffusion rate from the semisolid preparation when compared to free nanoparticles.

HVO presented the same profile. The amount of oil released was intermediate compared with NPVO and HNPVO, however. This result suggests the diffusion mechanism in hydrogel controls more significantly the oil release than its encapsulation in the developed nanosystem.

Cymbopogon citratus VO dissolution efficiency (DE) from NPVO was 70.68% ± 5.2, while HVO and HNPVO showed 63.64% ± 2.8 and 57.01% ± 2.9, respectively. This shows that the incorporation of nanoparticles in hydrogels resulted in a slow and gradual oil release when compared with the nanoparticulate form and free in hydrogels ($p < 0.05$, ANOVA).

The dissolution curves were fitted using different mathematical models and the results are presented in Table 3. By comparing the coefficients, the model proposed by Hixson-Crowell best described the oil release from NPVO, assuming the transport occurs due to the reduction of particle diameter by dissolution. The n analysis indicates an anomalous kinetic behavior (non Fickian) dependent on PLGA matrix erosion as well as on the diffusion mechanism. Similar kinetic behavior was reported by Chen et al. (2013) but it differs from most works about PLGA nanoparticles which describe the same non-Fickian release mechanism (Ibrahim et al., 2013; Khuroo et al., 2014).

HVO and HNPVO showed release profiles governed by a passive (Fickian) diffusion mechanism, as described by Higuchi (Costa and Lobo, 2001). The diffusion coefficient (n) of the Peppas model indicated an anomalous behavior dependent on both the oil diffusion from the polymeric network and the molecular relaxation of the polymer chains. In the present study, the effect of the gelling agent was more pronounced to control CcVO release.

Fontana et al. (2011) noted the release of clobetasol propionate from Carbopol® Ultrez nanocapsules occurred at a lower rate than nanoemulsions, following the Higuchi model. It suggests the importance of the gelling agent to control the drug release from the hydrogel.

The results obtained in the current study support the hypothesis that polymer matrix formed by the gelling agent could be the major responsible for minimizing the volatile oil evaporation.

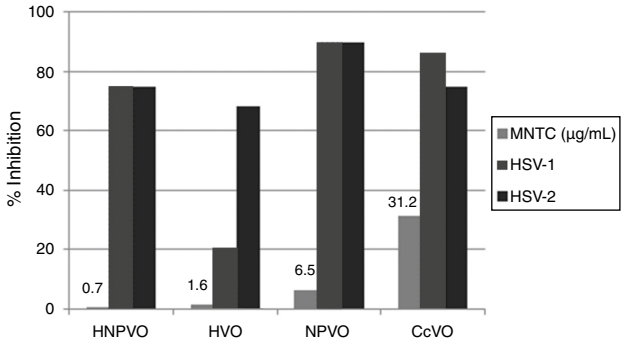

Fig. 3. Determination of the percentage inhibition of the samples against Herpes simplex types 1 and 2 in maximum non toxic concentrations (MNTC) in Vero cells using titer reduction assay.

Cytotoxicity and antiviral activity assays

The results given in Table 4 show the 50% cytotoxic concentration (CC_{50}) of oil (μg/ml) in HVO, HB, NPVO, HNPVO, NP formulations and free oil. In this study, 250 μg/ml of test sample were used as final formulation concentration, which means different CcVO content in tested samples. Incorporation of volatile oil in polymeric matrices decreased cytotoxicity against Vero cells, mainly with HNPVO, which showed lower MNTC (0.74 μg/ml).

Antiviral activity against HSV-1 and HSV-2 was investigated by viral titer reduction assay, employing previously determined MNTC from CcVO. All samples showed a very high inhibition potential against both viral types (Fig. 3). HNPVO efficiently inhibited

both viral strains in a very low concentration of CcVO 0.74 μg/ml (PI = 74.9%), 42.16, 8.76 and 2.23 times lower than free oil, NPVO and HVO, respectively.

Selectivity index (SI) for formulations (Table 4) is related to their safety in use. Results suggested the importance of volatile oil encapsulation in polymeric nanoparticles, demonstrated by a higher decrease in EC_{50} values when compared to free oil and loaded nanoparticles, 11.59 μg/ml to 1.32 μg/ml (HSV-1) and 6.69 μg/ml to 1.34 μg/ml (HSV-2), respectively. In some cases, SI could not be determined, because CC_{50} was higher than the initial concentration used in the cytotoxicity assay. Furthermore, when NP was incorporated in hydrophilic gel, a greater reduction in EC_{50} was observed. SI was more pronounced after encapsulation in polymeric matrices when considering the reduced oil content employed in the formulation.

Cymbopogon citratus VO incorporation in nanostructured systems provides greater area contact, favoring the interaction with the viral membrane. The small size of the particles provides a high oil content deposition on its surface, which tends to make it more bioavailable (Flores et al., 2013). Furthermore, polymeric network formed by Carbopol® Ultrez 10 NF gel seems to interfere in antiviral activity, presumably by modulating CcVO release from the formulation and protecting against volatilization. For this reason, HNPVO has greater activity against HSV in relation to NPVO and HVO. Similarly, nanogel biological activity was higher when compared to free oil, once a much lower CcVO concentration was used in the formulation.

Volatile oils and their major chemical constituents have been studied in order to identify new anti-herpes agents of natural origin (Bourne et al., 1999; Schuhmacher et al., 2003; Farag et al., 2004; Schnitzler et al., 2007, 2008; Koch et al., 2008). In a published work by Schnitzler and col. (2008), *Melissa officinalis* volatile oil showed strong virucidal activity against HSV-1 and -2, with EC_{50} values determined at high dilutions of 0.0004% and 0.00008%, respectively. These activities were attributed to its major constituent, citral and citronellal. Orhan et al. (2012) also observed inhibitory effect against Madin-Darby bovine kidney cell line infected with HSV-1 in MNTC for citral (3.2 μg/ml) and citronellal (1.6 μg/ml). These studies support the fact that citral must be primarily related to CcVO anti-herpetic activity or participate with other chemical compounds in a synergistic effect.

The significant increase in CcVO anti-herpetic action coming from their encapsulation in PLGA nanoparticles and subsequent incorporation into semisolid formulation seems to justify the use of nanotechnology in order to enhance the biological activity of substances from natural origin, such as volatile oils or their isolated constituents.

Conclusion

In this study, Carbopol® Ultrez hydrogel incorporated with *C. citratus* volatile oil encapsulated in PLGA nanoparticles was formulated. Nanoparticles were prepared by solvent emulsification–diffusion technique and vehiculated in hydrophilic gel. The oil content remained unchanged during the period evaluated. *In vitro* volatile oil release studies revealed a controlled release following Hixon-Crowell's model in PLGA nanoparticles and Higuchi's model in hydrogel. Moreover, the formulation exhibited anomalous behavior, governed by mechanisms of diffusion and polymeric erosion. In the evaluation of antiviral activity, nanogel was able to inhibit both viral strains in the non-cytotoxic oil concentration lower than the one observed in the hydrogel with free volatile oil.

Considering the greater protection from the volatile oil, its sustained release and the high selectivity index with reduced oil concentrations provided by incorporation into hydrogel, makes this a promising delivery system for topical herpes treatment.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

KBA contributed running the laboratory work, analysis of the data and drafted the paper. ACFA contributed in collecting plant sample and identification, and confection of herbarium. JLA contributed to development and characterization of the formulations. SCM contributed to *in vitro* CcVO release studies. JFC and MTVR contributed to antiviral activity studies. DQF designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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References

Abreu, F.O.M.S., Oliveira, E.F., Paula, H.C.B., Paula, R.C.M., 2012. Chitosan/cashew gum nanogels for essential oil encapsulation. Carbohydr. Polym. 89, 1277–1282.
Adams, R.P., 2007. Identification of Essential Oils Components by Gas Chromatography/quadrupole Mass Spectroscopy, 4th ed. Allured Publishing Corporation.
Anvisa, 2010. Farmacopeia Brasileira, 5a ed. Agência Nacional de Vigilância Sanitária, Ministério da Saúde, Brasília, DF, pp. 189–204.
Aiemsaard, J., Aiumlamai, S., Aromdee, C., Taweechaisupapong, S., Khunkitti, W., 2011. The effect of lemongrass oil and its major components on clinical isolate mastitis pathogens and their mechanisms of action on Staphylococcus aureus DMST 4745. Res. Vet. Sci. 91, e31–e37.
Astani, A., Reichling, J., Schnitzler, P., 2011. Screening for antiviral activities of isolated compounds from essential oils. Evid. Based Complem. Alternat. Med., http://dx.doi.org/10.1093/ecam/nep187.
Bernstein, D.I., Bellamy, A.R., Hook, E.W., Levin, M.J., Wald, A., Ewell, M.G., Wolff, P.A., Deal, C.D., Heineman, T.C., Dubin, G., Belshe, R.B., 2013. Epidemiology, clinical presentation, and antibody response to primary infection with herpes simplex virus type 1 and type 2 in young women. Clin. Infect. Dis. 56, 344–351.
Borenfreund, E., Puerner, J., 1985. Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol. Lett. 24, 119–124.
Bouchemal, K., Briançon, S., Perrier, E., Fessi, H., Bonnet, I., Zydowicz, N., 2004. Synthesis and characterization of polyurethane and poli (ether urethane) nanocapsules using a new technique of interfacial polycondensation combined of spontaneous emulsification. Int. J. Pharm. 269, 89–100.
Bourne, K.Z., Bourne, N., Reising, S.F., Stanberry, L.R., 1999. Plant products as topical microbicide candidates: assessment of in vitro and in vivo activity against herpes simplex virus type 2. Antiviral Res. 42, 219–226.
Chen, X., Peng, L.H., Shan, Y.H., Li, N., Wei, W., Yu, L., Li, Q.M., Liang, W.Q., Gao, J.Q., 2013. Astragaloside IV-loaded nanoparticle-enriched hydrogel induces wound healing and anti-scar activity through topical delivery. Int. J. Pharm. 447, 171–181.

Clogston, J.D., Patri, A.K., 2011. Zeta potential measurement. Methods Mol. Biol. 697, 63–70.
Cohen-Sela, E., Teitlboim, S., Chorny, M., Koroukhov, N., Danenberg, H.D., Gao, J., Golomb, G., 2009. Single and double emulsion manufacturing techniques of an amphiphilic drug in PLGA nanoparticles: formulations of mithramycin and bioactivity. J. Pharm. Sci. 98, 1452–1462.
Costa, P., Lobo, J.M.S., 2001. Modeling and comparison of dissolution profiles. Eur. J. Pharm. Sci. 13, 123–133.
Falcão, D.Q., Mourão, S.C., Araujo, J.L., Pereira, P.A.K, Cardoso, A.C.A., Almeida, K.B., Zibetti, F.M., Lima, B.G., 2015. Challenges in development of essential oil nanodelivery systems and future prospects. In: Naik, J. (Ed.), Nano Based Drug Delivery. IAPC Publishing, Zagreb, pp. 557–578.
Falcão, D.Q., Santos, A.R., Ortiz-Silva, B., Louro, R.P., Seiceira, R., Finotelli, P.V., Ferreira, J.L.P., De Simone, S.G., Amaral, A.C.F., 2011. Development of nanoencapsulation forms from *Cymbopogon citratus* essential oil. Lat. Am. J. Pharm. 30, 765–772.
Farag, R.S., Shalaby, A.S., El-Baroty, G.A., Ibrahim, N.A., Ali, M.A., Hassan, E.M., 2004. Chemical and biological evaluation of the essential oils of different *Melaleuca* species. Phytother. Res. 18, 30–35.
Fatahzadeh, M., Schwartz, R.A., 2007. Human herpes simplex virus infections: epidemiology, pathogenesis, symptomatology, diagnosis, and management. J. Am. Acad. Dermatol. 57, 737–763.
Fernandes, C.P., Mascarenhas, M.P., Zibetti, F.M., Lima, B.G., Oliveira, R.P.R.F., Rocha, L., Falcão, D.Q., 2013. HLB value, an important parameter for the development of essential oil phytopharmaceuticals. Rev. Bras. Farmacogn. 23, 108–114.
Flores, F.C., Lima, J.A., Ribeiro, R.F., Alves, S.H., Rolim, C.M.B., Beck, R.C.R., Silva, C.B., 2013. Antifungal activity of nanocapsule suspensions containing tea tree oil on the growth of *Trichophyton rubrum*. Mycopathologia 175, 281–286.
Fontana, M.C., Rezer, J.F.P., Coradini, K., Leal, D.B.R., Beck, R.C.R., 2011. Improved efficacy in the treatment of contact dermatitis in rats by a dermatological nanomedicine containing clobetasol propionate. Eur. J. Pharm. Biopharm. 79, 241–249.
Förster, M., Bolzinger, M.A., Fessi, H., Briançon, S., 2009. Topical delivery of cosmetics and drugs. Molecular aspects of percutaneous absorption and delivery. Eur. J. Dermatol. 19, 309–323.
Gomes, C., Moreira, R.G., Castell-Perez, E., 2011. Poly (DL-lactide-co-glycolide) (PLGA) nanoparticles with entrapped trans-cinnamaldehyde and eugenol for antimicrobial delivery applications. J. Food Sci. 76, 16–24.
Guterres, S.S., Alves, M.P., Pohlmann, A.R., 2007. Polymeric nanoparticles, nanospheres and nanocapsules, for cutaneous applications. Drug Target Insights 2, 147–157.
Iannitelli, A., Grande, R., Di Stefano, A., Di Giulio, M., Sozio, P., Bessa, L.J., Laserra, S., Paolini, C., Protasi, F., Cellini, L., 2011. Potential antibacterial activity of carvacrol-loaded poly (DL-lactide-co-glycolide) (PLGA) nanoparticles against microbial biofilm. Int. J. Mol. Sci. 12, 5039–5051.
Ibrahim, M.M., Abd-Elgawad, A.E.H., Soliman, O.A.E., Jablonski, M.M., 2013. Pharmaceutical nanotechnology nanoparticle-based topical ophthalmic formulations for sustained celecoxib release. J. Pharm. Sci. 102, 1036–1053.
Jeong, Y., Na, H., Seoa, D., Kim, D., Lee, H., Jang, M., Na, S., Roh, S., Kim, S., Nah, J., 2008. Ciprofloxacin-encapsulated poly (DL-lactide-co-glycolide) nanoparticles and its antibacterial activity. Int. J. Pharm. 352, 317–323.
Kahan, M.T.H., Ather, A., Thompson, K.D., Gambari, R., 2005. Extracts and molecules from medicinal plants against herpes simplex viruses. Antiviral Res. 67, 107–119.
Khuroo, T., Verma, D., Talegaonkar, S., Padhi, S., Panda, A.K., Iqbal, Z., 2014. Topotecan–tamoxifen duple PLGA polymeric nanoparticles: investigation of in vitro, in vivo and cellular uptake potential. Int. J. Pharm. 473, 384–394.
Koch, C., Reichling, J., Schneele, J., Schnitzler, P., 2008. Inhibitory effect of essential oils against herpes simplex virus type 2. Phytomedicine 15, 71–78.
Markoulatos, P., Georgopoulou, A., Siafakas, N., Plakokefalos, E., Tzanakaki, G., Kourea-Kremastinou, J., 2001. Laboratory diagnosis of common herpes virus infections of the central nervous system by a multiplex PCR assay. J. Clin. Microbiol. 39, 4426–4432.
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. Microbiol. Immun. 47, 681–684.
Moinard-Chécot, D., Chevalier, Y., Briançon, S., Beney, L., Fessi, H., 2008. Mechanism of nanocapsules formation by the emulsion-diffusion process. J. Colloid. Interface Sci. 317, 458–468.
Mora-Huertas, C.E., Fessi, H., Elaissari, A., 2010. Polymer-based nanocapsules for drug delivery. Int. J. Pharm. 385, 113–142.
Mu, L., Feng, S.S., 2003. PLGA/TPGS Nanoparticles for controlled release of paclitaxel: effects of the emulsifier and drug loading ratio. Pharm. Res. 20, 1864–1872.
Naik, M.I., Fomda, B.A., Jaykumar, E., Bhat, J.A., 2010. Antibacterial activity of lemongrass (*Cymbopogon citratus*) oil against some selected pathogenic bacteria. Asian Pac. J. Trop. Med. 3, 535–538.
Negrelle, R.R.B., Gomes, E.C., 2007. *Cymbopogon citratus* (DC.) Stapf: chemical composition and biological activities. Rev. Bras. Pl. Med. 9, 80–92.
Nishimura, T., Toku, K., Fukuyasu, H., 1977. Antiviral compounds XII. Antiviral activity of amino hydrazones of alkoxyphenyl substituted carbonyl compounds against influenza virus in eggs and mice. Kitasato Arch. Exp. Med. 50, 39–46.
Oloyede, O.I., 2009. Chemical profile and antimicrobial activity of *Cymbopogon citratus* leaves. J. Nat. Prod. 2, 98–103.
Orhan, I.E., Özçelik, B., Kartal, M., Kan, Y., 2012. Antimicrobial and antiviral effects of essential oils from selected Umbelliferae and Labiatae plants and individual essential oil components. Turkish J. Biol. 36, 239–246.
Pereira, P.P., Puntel, R.L., Boschetti, T.K., Morel, A.F., 2009. Antioxidant effects of different extracts from *Melissa officinalis*, *Matricaria recutita* and *Cymbopogon citratus*. Neurochem. Res. 34, 973–983.
Pereira, R.S., Sumita, T.C., Furlan, M.R., Jorge, A.O.C., Ueno, M., 2004. Atividade antibacteriana de óleos essenciais em cepas isoladas de infecção urinária. Rev. Saúde Pública 38, 326–328.
Pinto, Z.T., Sánchez, F.F., Santos, A.R., Amaral, A.C.F., Ferreira, J.L.P., Escalona-Arranz, J.C., Queiroz, M.M.C., 2015. Chemical composition and insecticidal activity of *Cymbopogon citratus* essential oil from Cuba and Brazil against housefly. Braz. J. Vet. Parasitol. 24, 36–44.
Reed, L.J., Müench, H., 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27, 493–497.
Rodriguez, D.J., Chulia, J., Simões, C.M.O., Amoros, M., Mariotte, A.M., Girre, L., 1990. Search for in vitro antiviral activity of a new isoflavonic glycoside from Ulex europaeus. Planta Med. 56, 59–62.
Sahana, D.K., Mittal, G., Bhardwaj, V., Ravi Kumar, M.N.V., 2008. PLGA Nanoparticles for oral delivery of hydrophobic drugs: influence of organic solvent on nanoparticle formation and release behavior in vitro and in vivo using estradiol as a model drug. J. Pharm. Sci., 1530–1542.
Sahoo, S.K., Panyam, J., Prabha, S., Labhasetwar, V., 2002. Residual polyvinyl alcohol associated with poly (DL-lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake. J. Control. Release 82, 105–114.
Schaffazick, S.R., Guterres, S.S., 2003. Caracterização e estabilidade físico-química de sistemas poliméricos nanoparticulados para administração de fármacos. Quim. Nova 26, 726–737.
Schaneberg, B.T., Khan, I.A., 2002. Comparison of extraction methods for marker compounds in the essential oil of lemon grass by GC. J. Agric. Food Chem. 50, 1345–1349.
Schnitzler, P., Koch, C., Reichling, J., 2007. Susceptibility of drug-resistant clinical herpes simplex virus type 1 strains to essential oils of ginger, thyme, hyssop, and sandalwood. Antimicrob. Agents Chemother. 51, 1859–1862.
Schnitzler, P., Schuhmacher, A., Astani, A., Reichling, J., 2008. Melissa officinalis oil affects infectivity of enveloped Herpes viruses. Phytomedicine 15, 734–740.
Schuhmacher, A., Reichling, J., Schnitzler, P., 2003. Virucidal effect of peppermint oil on the enveloped viruses herpes simplex virus type 1 and type 2 in vitro. Phytomedicine 10, 504–510.
Silva, C.B., Guterres, S.S., Weisheimer, V., Schapoval, E.E.S., 2009. Antifungal activity of the lemongrass oil and citral against *Candida* spp. Braz. J. Infect. Dis. 12, 63–66.
Tajidin, N.E., Ahamad, S.H., Rosenani, A.B., Azimah, H., Munirah, M., 2012. Chemical composition and citral content in lemongrass (*Cymbopogon citratus*) essential oil at three maturity stages. Afr. J. Biotechnol. 11, 2685–2693.
Thompson, K.D., 2006. Herbal extracts and compounds active against herpes simplex virus. Adv. Phytomed. 2, 65–86.
United States Pharmacopeia (USP), 2008. USP 31; The National Formulary: NF 26, vol. 1. United States Pharmacopeial Convention, Rockville, pp. 817.
Viana, G.S.B., Vale, T.G., Pinho, R.S.N., Matos, F.J.A., 2000. Antinociceptive effect of the essential oil from *Cymbopogon citratus* in mice. J. Ethnopharmacol. 70, 323–327.
Weisheimer, V., Miron, D., Silva, C.B., Guterres, S.S., Schapoval, E.E.S., 2010. Microparticles containing lemongrass volatile oil: preparation, characterization and thermal stability. Pharmazie 65, 885–890.
Wu, L., Zhang, J., Watanabe, W., 2011. Physical and chemical stability of drug nanoparticles. Adv. Drug Deliv. Rev. 63, 456–469.
Zhang, T., Sturgis, T.F., Youan, B.C., 2011. pH-responsive nanoparticles releasing tenofovir intended for the prevention of HIV transmission. Eur. J. Pharm. Biopharm. 79, 526–536.