Natural membranes of *Hevea brasiliensis* latex as delivery system for *Casearia sylvestris* leaf components

Flávio A. Carvalho, Helena S. Uchina, Felipe A. Borges, Márcia H. Oyafuso, Rondinelli D. Herculano, Maria P.D. Gremião, André G. Santos

**A R T I C L E  I N F O**

Article history:
Received 9 May 2017
Accepted 10 October 2017
Available online 6 December 2017

Keywords:
Diterpenes
Casearin
Phenolic compounds
In vitro permeation
In vitro retention
Natural latex membrane

**A B S T R A C T**

Natural latex from *Hevea brasiliensis* (Wild. ex A.Juss) Müll.Arg., Euphorbiaceae, showed angiogenic action and *Casearia sylvestris* Sw., Salicaceae, leaf derivatives presented anti-inflammatory and wound healing activities. Therefore, an association of these effects was interesting for wound healing applications. The aims of this study were the development of membranes of natural latex incorporated with *C. sylvestris* leaf derivatives (ethanolic extract, diterpene concentrated fraction and casearin); their chemical and physical characterization, and the evaluation of in vitro skin permeation and retention of *C. sylvestris* bioactive secondary metabolites (diterpenes and phenolic compounds). The membranes were developed mixing hydroethanolic solutions of *C. sylvestris* derivatives with latex and drying them in a desiccator. They were characterized by infrared spectroscopy, scanning electron microscopy, water vapor permeability and mechanical resistance assays, demonstrating that all membranes were permeable, resistant and homogeneous in surfaces. The permeation and retention assays demonstrated dermal penetration of phenolic compounds for ethanolic extract membrane and of casearin-like clerodane diterpenes for all membranes, indicating that these membranes have great potential for therapeutic application as a topical system for *C. sylvestris* components releasing.

© 2017 Sociedade Brasileira de Farmacognosia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

**Introduction**

*Hevea brasiliensis* (Wild. ex A.Juss) Müll.Arg., Euphorbiaceae, is a tree native from the Amazon rainforest, known in Brazil as “seringueira” (Priyadarshan and Clement-Demange, 2004). In addition to its various commercially exploited properties, the latex extracted from *H. brasiliensis* accelerates the angiogenesis process (Frade et al., 2004; Herculano et al., 2009) acting in tissue neoformation (Alves de Sousa et al., 2007; Balabanian et al., 2006) and regeneration (Oliveira et al., 2003). Natural latex membranes (NLMs) have been applied in bedsores and burns (Andrade et al., 2011), neoformation of tympanic membrane (Oliveira et al., 2003), chronic ulcers, femoral arterioplasty, pericardium replacement in dogs, reconstruction of the conjunctiva in rabbits and tympanic membrane regeneration (Alves de Sousa et al., 2007; Balabanian et al., 2006).

The NLMs were also evaluated as a matrix in drug delivery (Herculano et al., 2007) incorporated with metronidazole (Herculano et al., 2010), ciprofloxacin (Murbach et al., 2014), diclofenac (Aielo et al., 2014), *Stryphnodendron adstringens* (Mart.) Coville stem bark extract (Romeia et al., 2012) and *Casearia sylvestris* Sw. ethanolic leaf extract (Bolognesi et al., 2015; Borges et al., 2014; Trecco et al., 2014). Other authors reported the permeation of nicotine incorporated in NLM (Pichayakorn et al., 2012a,b).

*C. sylvestris* Sw., Salicaceae, is a tree or shrub from Central and South America. In Brazil, it is known as “guacatonga”, “erva-de-lagarto”, and “cafezinho-do-mato” (Ferreira et al., 2011). The bioactive secondary metabolites in the species include terpenes (monoterpenses, sesquiterpenes and diterpenes) and phenolic compounds (ellagic acid derivatives and flavonoids). The casearin-like clerodane diterpenes are chemical markers for *Casearia* genus (Bueno et al., 2016; Ferreira et al., 2011; Pierri, 2013). Their

https://doi.org/10.1016/j.bjp.2017.10.007
0102-695X/© 2017 Sociedade Brasileira de Farmacognosia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
leaves are employed in traditional medicine as antiophilic, anti-inflammatory, antihemorrhagic, wound healing, and in the treatment of gastric disorders (Ferreira et al., 2011). Pharmacological studies demonstrated antiphilic, antitumor, antiulcerogenic, anti-inflammatory and wound healing actions of its extracts (ethanolic, hydroethanolic and aqueous extracts) and isolated compounds (Albano et al., 2013; Ferreira et al., 2011; Oshima-Franco et al., 2005; Pierri et al., 2017; Santos et al., 2010), relating the casearin-like clerodane diterpenes to the anti-inflammatory and wound healing actions (Mattos et al., 2007; Pierri, 2013; Pierri et al., 2017). Ellagic acid derivatives from C. sylvestris leaves exhibited anti-PLA2 activity and may be also related to C. sylvestris anti-inflammatory activity (Silva et al., 2008). Regarding the toxicity, C. sylvestris extracts and isolated diterpenes showed low toxicity both in vitro and in vivo (Basile et al., 1990; Ferreira et al., 2011; Maestro et al., 2004; Santos et al., 2010). An association of leaf derivatives from C. sylvestris (ethanolic extract and its fractions) or its isolated compounds with NLM may lead to a delivery system with angiogenic plus anti-inflammatory and wound healing properties. NLM incorporated with ethanolic extract of C. sylvestris have already been evaluated for the release of components in water and analyzed by SEM and FT-IR. The fast initial release (about 1 day) of C. sylvestris compounds (phenolic compounds and diterpenes) from NLM was attributed to the presence of the extract in the membrane surface and the slower release (35–40 days) was related to the diffusion of the compounds through the NLM. Different pH values (6.4–7.6) were evaluated in phenolic compounds water release and at 7.2 and 7.6 pH values the release was higher (Bolognesi et al., 2015; Borges et al., 2014; Trecco et al., 2014). However, in vitro skin permeation and retention assays are needed for human topical application of these membranes because they provide more significative data on skin drug delivery than water release models (Sato et al., 2007). Moreover, there are no studies with latex membranes incorporated with isolated compounds from C. sylvestris.

Thus, the main objectives of this study were the development of NLM associated with derivatives from the leaves of C. sylvestris (ethanolic extract, concentrated diterpene fraction and casearin J (1)) and the evaluation of in vitro skin permeation and retention of bioactive secondary metabolites of C. sylvestris (diterpenes and phenolic compounds). The physical and chemical characterization of the membranes were also performed.

Materials and methods

Phytochemistry

Leaves of Casearia sylvestris Sw., Salicaceae, were collected at Medicinal Botanical Garden of School of Pharmaceutical Sciences of São Paulo State University, Araraquara-SP (coordinates: “21°8′46.6″S, “48°20′21.5″W”), under license n. 010011/2015-3 of Genetic Heritage Management Council (CGEN), in October 2013 and identified by Dr. Luis V. S. Sacramento, UNESP Araraquara. A voucher specimen (AGS 102) was deposited at the Herbarium Maria E. P. K. Fidalgo of the Botanical Institute of São Paulo, Brazil.

The phytochemical procedures were performed according to Santos et al. (2010) with some modifications. Dried and powdered leaves of C. sylvestris (1.5 kg) were extracted by static maceration at 40 ºC (Tecnal®, TE-184 thermostated bath) with absolute ethanol (22.51, 96 h), and extractive solution was concentrated under reduced pressure at 40 ºC (IKA®, DEST-KV 05S3 rotaevaporator) and dried in a desiccator (silica gel) to yield 158 g of dry extract. Ethanolic dry extract (59 g) was fractionated by solid phase extraction (SPE) in a glass column (20 cm × 10 cm) with silica gel (63–200 μm Merck®) plus active charcoal (Synth®) 1:1 (w/w) using as mobile phase hexane: ethyl acetate 95:5, ethyl acetate and methanol to yield three fractions (SPE 1, 3.6 g; SPE 2, 10.7 g and SPE 3, 16.2 g). SPE 2 fraction (4 g) was submitted to normal-phase low pressure column chromatography in a glass column (20 cm × 5 cm) with silica gel (40–60 μm Merck®) resulting in 55 fractions which were analyzed by TLC (Claudino et al., 2013) and HPLC-PDA under the following conditions: Perkin Elmer® Flexar HPLC-PDA; Hypersil® Gold C18 column (250 mm × 4.6 mm, 5 μm); methanol: water 70:30 for 50 min; flow rate of 0.7 ml/min; sample injection volume of 20 μl (1 mg/ml, methanol); detection at 235 nm (210–400 nm). HPLC sample pretreatment was performed as described by Claudino et al. (2013). Fractions 12–14 (369.8 mg) from CC were purified through preparative HPLC under the following conditions: Shimadzu® preparative HPLC-PDA (pump LC-GAD, system control CBM-20A, detector SPD-M20A); Phenomenex® Luna C18 column (250 mm × 21.0 mm, 10 μm); methanol: water 65:35 for 70 min; flow rate of 15.0 ml/min; sample injection volume of 2 ml (20 mg/ml, methanol: water 85:15); detection at 235 nm (210–350 nm). Collected peak: tR = 57.0 min (casearin J). The chromatographic purity of casearin J (1) was determined as 98.0% (235 nm) using the same HPLC-PDA analytical method described above.

Casearin J spectrum were obtained in a Bruker® spectrometer (1H 300 MHz, 13C 75 MHz) – 7.0 T in pulse-gradient mode. Pyridine-d5 was used as solvent (20 mg/ml). IR spectrum was obtained in a Bruker Tensor® 27 ATR FT-IR spectrometer using 1 mg of cas J: 4000–500 cm–1, 64 scans, resolution of 4 cm–1. UV spectrum was obtained in a Shimadzu®UV-1800 spectrophotometer with absolute ethanol as solvent (0.1 mg/ml). ESI-MS was performed using a LTQ linear ion trap (Thermo Fisher Scientific®, San Jose, CA, USA) mass spectrometer in the positive ion mode. The sample was dissolved in ethanol (1 μg/ml) and directly infused through the syringe pump of LTQ. The main parameters adopted were the following: nitrogen as nebulizing gas (100 psi), spray solvent flow rate of 5 μl/min, spray voltage of 5.0 kV, capillary voltage of 10.0 V and capillary temperature of 285 ºC. Full scan spectrum was acquired in the range of m/z 200 to 800 and accumulated for 60 s.

NMR spectra were obtained in a Bruker® spectrometer (~1H 300 MHz, 13C 75 MHz) – 7.0 T in pulse-gradient mode. Pyridine-d5 was used as solvent (20 mg/ml). IR spectrum was obtained in a Bruker Tensor® 27 ATR FT-IR spectrometer using 1 mg of cas J: 4000–500 cm–1, 64 scans, resolution of 4 cm–1. UV spectrum was obtained in a Shimadzu®UV-1800 spectrophotometer with absolute ethanol as solvent (0.1 mg/ml). ESI-MS was performed using a LTQ linear ion trap (Thermo Fisher Scientific®, San Jose, CA, USA) mass spectrometer in the positive ion mode. The sample was dissolved in ethanol (1 μg/ml) and directly infused through the syringe pump of LTQ. The main parameters adopted were the following: nitrogen as nebulizing gas (100 psi), spray solvent flow rate of 5 μl/min, spray voltage of 5.0 kV, capillary voltage of 10.0 V and capillary temperature of 285 ºC. Full scan spectrum was acquired in the range of m/z 200 to 800 and accumulated for 60 s.

Phenolic compounds and diterpenes quantification

Photometric methods: phenolic compounds were quantified in extract and in extract membrane at 269 nm employing an analytical curve of rutin (Merck®, 97% purity) with solutions at concentrations of 0.63, 1.25, 2.5, 5.0, 7.5, 10.0, 15.0, 20.0 μg/ml (absolute EtOH).
Quantification of diterpenes in membranes was realized at 235 nm employing an analytical curve of casearin J (0.63, 1.25, 2.5, 5.0, 7.5, 10.0, 15.0, 20.0, 25.0, 30.0, 40.0 μg/ml). All photometric analysis were performed in Shimadzu® UV-1800 spectrophotometer.

**HPLC method:** Total diterpenes and casearin J (1 quantitation in extract and SPE 2 was realized as described by Claudino et al. (2013) in a Perkin Elmer® Flexar HPLC-PDA analytical curve of casearin J (98.0% purity at 235 nm; 0.025, 0.0375, 0.05, 0.075, 0.1, 0.15, 0.3 mg/ml; MeOH). The cerodane diterpenes casearin B and caseargewin F purified by Santos et al. (2010) were also used as standards (1 mg/ml; MeOH) in this HPLC analysis for identification purposes.

**Membrane production**

The latex of *Hevea brasiliensis* was acquired from BDF Latex Company (Guarantã-SP, Brazil) and it was processed in order to reduce the concentration of allergenic proteins (Herculano et al., 2009). Membranes were produced in Petri dishes (6 cm; total area = 28.27 cm²) using 6 ml of latex and ethanol/water 4:6 solutions of *C. sylvestris* derivatives (Table 1). All membranes were dried for 66 h in a desiccator (silica gel). NLM was also dried under UV light for comparison purposes. Contents of total phenolics and diterpenes in NLM Extract and NLM SPE 2 (only diterpenes) were calculated considering amounts of extract and SPE 2 added to membranes and the determination of phenolic compounds and diterpenes in these *C. sylvestris* derivatives according to phenolic compounds and diterpenes quantification (“Materials and methods” section).

**Membrane characterization**

Extract, SPE 2, cas J (1 mg) and membrane surfaces were analyzed in a Bruker® Tensor 27, ATR FT-IR spectrometer: 4000–500 cm⁻¹, 64 scans, with a resolution of 4 cm⁻¹ (Murbach et al., 2014). Surface morphology was evaluated by scanning electron microscopy under reduced pressure with 2.000 zoom (FEG-SEM JRL® 7500F) (Herculano et al., 2009). Mechanical strength testing was applied in membranes (7.5 mm × 2.5 mm × 0.9 mm) by mechanical testing machine (EMIC® DL 2000) with 10 kgf load cell at 500 mm/min (according to ASTM D412) (Murbach et al., 2014). Water vapor permeability (WVP) and water vapor transmission rate (WVTR) of membranes (1.66 cm²) were measured at times 0, 24, 48, 72, 96, 120 h and calculated according to literature (Prezotti et al., 2012). Membrane thickness was measured with a micrometer (Western®, 0–12.7 mm). All experiments for membrane characterization were performed with three membranes of each type (NLM, extract, SPE 2 and cas J membranes) and results were expressed as mean of triplets and standard deviation.

In *vitro* permeation and skin retention

Porcine ear skin obtained from a slaughterhouse (Olhos d’água, Ipuã-SP, Brazil) was dissected and dermatomed (NOUVAG® Dermatome) providing skin pieces with 500 μm thickness. In *vitro* permeation assays of membranes (NLM, NLM Extract, NLM SPE2 and NLM J) were performed using a Franz’s cell apparatus (Hanson Microrette® HANSON 0700-1251) containing six modified Franz’s cells and using six samples of 1.77 cm² from each type of membrane. Porcine ear skin was placed between donor compartment (7 ml) and Franz’s cell receptor, keeping stratum corneum in contact with membranes and dermis in contact with receptor solution (ultrapure water). Assays were developed at constant magnetic stirring (300 ± 0.2 rpm) and 37 ± 0.5°C (Boakye et al., 2015; Dvivedi et al., 2016). NLM Extract presented 45.2 μg/cm² of phenolic compounds; NLM Extract, NLM SPE 2 and NLM J presented 58.2, 43.2 and 39.9 μg/cm² of diterpenes, respectively ("Membrane production” section).

Aliquots (2 ml) were collected automatically after 0.5, 1, 2, 4, 6, 8, 12, 18 and 24 h. Permeation values of diterpenes and total phenolics were determined from aliquots in an UV spectrophotometer (Shimadzu® UV-1800) at 235 nm and 269 nm employing analytically curves of cas J and rutin (“Phenolic compounds and diterpene quantification” section) to quantify diterpenes and total phenolics, respectively. Ultrapure water was blank in these determinations. Collected aliquots were automatically replaced and calculations considered dilutions after the first collect using equation, \( Q_{rel} = \frac{C_{t}V_{t} + \sum C_{i}V_{i}}{C_{rel}} \), where: \( Q_{rel} = \) real quantity relative to time t; \( C_{i} = \) concentration obtained relative to time t; \( V_{i} = \) receptor solution volume (7 ml); \( C_{r} = \) concentration of previous sampling; \( V_{s} = \) sampled (collected + dead volume), according to US Pharmacopoeia XXIX (2006).

Permeation data were plotted by software SigmaPlot® 10.0 and submitted to ANOVA statistical analysis. Results were expressed as mean of sextuplicate and standard deviation. Math models (Table 2) relative to permeation profiles were fitted using software SigmaPlot® 10.0.

After permeation assays, skin was removed from the Franz’s cell and stratum corneum was separated using tape stripping technique. Skin was tape-stripped 16 times using adhesive tapes (Scotch® 750 3 M) discarding first tape. Tape strips were added in tube with 5 ml of methanol, stirred in vortex (PHOENIX® AP 56, 3800 rpm) for 2 min, following by bath sonication (Unitec® USC 2800, 40 kHz) for 30 min. Epidermis and dermis were cut with scissors, added in tube with 5 ml of methanol, stirred in vortex for 2 min, homogenized in ultra-turrax (IKA® T 18) for 2 min, following bath sonication for 30 min (Bianchi et al., 2016; Boakye et al., 2015). Stratum corneum, epidermis and dermis sample solutions were filtered in 0.22 μm membranes (PVDF Millipore®) and analyzed through UV photometry to quantify diterpenes.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Volume of hydroethanolic solution (ml)</th>
<th>Incorporated derivative (mg)</th>
<th>Diterpenes content per membrane (mg)</th>
<th>Total phenolic compounds content per membrane (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLM</td>
<td>5</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NLM Extract</td>
<td>5</td>
<td>Extract (20.0)</td>
<td>2.9</td>
<td>2.4</td>
</tr>
<tr>
<td>NLM SPE 2</td>
<td>1</td>
<td>SPE 2 (4.0)</td>
<td>2.1</td>
<td>–</td>
</tr>
<tr>
<td>NLM J</td>
<td>cas J (2.0)</td>
<td>2.0</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

**Table 1**

Data on membrane production.

**Table 2**

Models of applied math calculations.

<table>
<thead>
<tr>
<th>Model</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baker-Lonsdale</td>
<td>( C_{t} = \frac{Q}{F} ) \times t</td>
</tr>
<tr>
<td>First-order</td>
<td>( F = \frac{A}{V_{t}} ) \times t</td>
</tr>
<tr>
<td>Higuchi</td>
<td>( F = \frac{Q}{C_{t}} ) \times t</td>
</tr>
<tr>
<td>Hixon</td>
<td>( F = \frac{Q}{C_{t}} ) \times t</td>
</tr>
<tr>
<td>Peppas</td>
<td>( F = \frac{Q}{C_{t}} ) \times t</td>
</tr>
<tr>
<td>Weibull</td>
<td>( m = 1 - \exp \left( \frac{-t}{N} \right) )</td>
</tr>
</tbody>
</table>

* F = amount of drug released at time t; \( k_{w}, k_{r}, k_{d}, k_{o} \) and \( k_{p} \) = release rate constants for the different equations; \( n \) = release exponent.
and total phenolics according to in vitro permeation methods described above and "Phenolic compounds and diterpene quantification" section. Porcine ear skin control (blank) was submitted to permeation/retention assays and obtained solutions did not show absorbance at 235 and 269 nm. Retention data were expressed as mean of sextuplicate and standard deviation. Data were also submitted to ANOVA statistical analysis.

Results and discussion

Casearin J identification

Casearin J was identified on the basis of NMR, FT-IR, UV and ESI-MS spectrometric data besides comparison with literature data (Morita et al. 1991).

Casearin J: white powder. UV (ethanol): \( \lambda_{	ext{max}} \) 234 nm (log \( \varepsilon \) 3.69). ATR FT-IR: 3460, 3092, 2932, 2876, 1748, 1690, 1463, 1369, 1219, 1066, 1005, 983, 918 cm\(^{-1}\). ESI-MS (positive ion mode): m/z 601 [M+K]+ (100%), 585 [M+Na]+ (26%), 580 [M+NH4]+ (8%). \(^1\)H NMR (pyridine-d5, 300 MHz): \( \delta \) 7.44, 7.26, 6.67, 6.39, 5.66, 5.55, 5.22, 5.08, 4.26, 3.97, 3.39, 2.68, 2.79, 2.35, 2.32, 2.17, 2.08, 2.02, 1.91, 1.73, 1.67, 1.59, 1.03, 0.97, 0.89, 0.83. \(^{13}\)C NMR (pyridine-d5, 75 MHz): \( \delta \) 173.8, 173.6, 169.9, 144.6, 134.4, 134.2, 127.7, 123.6, 114.9, 99.2, 97.3, 76.5, 74.8, 73.3, 57.0, 55.1, 41.9, 40.2, 37.0, 36.9, 36.8, 31.0, 26.6, 26.1, 21.9, 20.5, 19.2, 19.0, 14.2, 14.0, 11.9.

Phenolic compounds and diterpene quantification

Phenolic compounds and diterpene contents were determined in C. sylvestris derivatives and membranes because diterpenes showed anti-inflammatory and wound healing activity and phenolic compounds may be related to anti-inflammatory activity of C. sylvestris (Pierri, 2013; Pierri et al., 2017; Silva et al., 2008).

Total phenolic compounds were determined in extract as 11.9% (w/w) using analytical curve of rutin at 269 nm (equation: \( y = 37.455 \times + 0.0052; R^2 = 0.9997 \)). Thus, determination of total phenolic compounds in skin permeation and retention assays was performed using analytical curve of rutin (269 nm). This flavonoid was selected as external standard because fourteen glycosylated flavonoids were identified in the leaves of C. sylvestris, including rutin (Bueno et al., 2016). Regarding diterpene determination through HPLC-PDA (235 nm; equation: \( y = 28,018,592.8 \times + 107,798.6; R^2 = 0.9998 \)) extract and SPE 2 showed 0.5% and 6.5% (w/w) of cas J \((t_g = 19.30 \text{ min})\) and 14.6% and 54.3% (w/w) of total casearin-like diterpenes (Claudino et al., 2013), respectively. Extract, SPE 2 and cas J chromatograms are presented in Fig. 1.
Casearin B and caseargrewiin F ($t_R = 18.07$ and 15.52 min, respectively) were also identified in extract and SPE 2 on the basis of $t_R$ and UV spectra ($\lambda_{max} = 234$ nm) comparison.

**Membrane characterization**

The upper and lower surfaces of membranes were analyzed by attenuated total reflectance Fourier transform infrared (ATR FT-IR) in three distinct regions of each surface. With respect to drying methods, the FT-IR spectra of NLM (Fig. 2) and NLM Extract presented absorption bands between 2367 and 2355 cm$^{-1}$ only for membranes dried under UV light. These bands can be assigned to isocyanate or nitrile groups. As the natural latex has nitrogenated compounds as amino acids and proteins (Agostini et al., 2008), these bands suggest a reaction induced by UV light during drying. Even though UV light is germicide, it can induce photo oxidation by free radical mechanisms and the most photo reactive functional groups are carbonyls, nitro aromatics, N-oxides, alkenes, and aryl chlorides (Ahuja and Scypinski, 2001). Membranes dried at desiccator showed the same IR bands in the spectra for the three different regions of both surfaces, however for the membranes dried under UV light the surfaces did not show homogeneity. Based on these results, the membranes were dried in desiccator to avoid alterations in their components.

In addition, results of FT-IR analysis proved that membranes dried in desiccator under visible light or without exposure to visible light presented homogeneous upper and lower surfaces, indicating that the visible light did not interfere in membranes chemical composition.

Extract, SPE 2 and cas J were also analyzed by ATR FT-IR. The absorption band of carbonyl was only present in the extract, SPE 2 and cas J without latex (1741, 1736 and 1746 cm$^{-1}$, respectively), result similar to data previously described in literature (Bolognesi et al., 2015; Borges et al., 2014). The absorption band observed for NLM at 842–840 cm$^{-1}$, typical of a trisubstituted alkene, was attributed to poly-1,4-cis-isoprene, present in the natural latex and thus in all developed membranes. In extract, SPE 2 and cas J spectra the absorptions bands of carbonyl and single carbon-oxygen bonds of diterpenes were very, but when these derivatives were incorporated into NLM carbonyl bands were not observed. These absorption bands decreasing may be related to low concentration of diterpenes in membranes.

The analysis by scanning electron microscopy (SEM) of the membrane surfaces (upper and lower) allowed verifying the presence of particles only in NLM Extract, NLM SPE 2 and NLM J. As expected, NLM did not show particles on its surfaces. These data indicated that the observed particles correspond to components of C. sylvestris which can be released directly from the surface of the membranes. SEM also suggested morphological and structural uniformity in the membrane surfaces (Fig. 3). These results were coherent with literature data (Bolognesi et al., 2015; Borges et al., 2014).

The obtained values of water vapor transmission rate (WVTR) (Table 3 and Fig. 4) showed that all membranes were permeable. NLM J and NLM Extract presented the highest WVTR values. The thickness did not increase the permeability (Table 3). NLM water vapor permeability (WVP) showed the lower value among tested membranes. These results demonstrated that C. sylvestris components interacted with polymer chains of NLM converting it into a more permeable system.

Membranes incorporated with C. sylvestris components were more resistant to deformation and break than NLM (Fig. 5), showing that incorporated components in the natural latex increased the membranes breaking strain. The percentage of elongation at break of membranes was $941.0 \pm 10.2$ for NLM, $1147.0 \pm 17.1$ for NLM Extract, $1119.0 \pm 16.8$ for NLM SPE 2 and $1305.0 \pm 21.1$ for NLM J. Thickness of membranes (Table 3) and the interaction among NLM and C. sylvestris components may be factors responsible for resistance to deformation and break.

**In vitro permeation and skin retention**

**In vitro** permeation and skin retention studies were carried out to analyze interactions between developed membrane and skin. Several factors can influence skin permeation of active compounds such as drug diffusion through different skin layers (Berard et al., 2003; Freitas et al., 2015).

Contents of phenolic compounds and diterpenes in membranes evaluated in permeation and skin retention assay were calculated as: 45.2 µg/cm² of phenolic compounds for NLM Extract; 58.2, 43.2 and 39.9 µg/cm² of casearin-like clerodane diterpenes for NLM Extract, NLM SPE 2 and NLM J, respectively. Fig. 6D demonstrates that phenolic compounds of NLM Extract beginning the permeation after 4h. Permeated value found into the receptor solution after 24 h was 3.96±0.50 (w/w). Obtained results for diterpenes demonstrated a distinct performance; permeation beings at the first 30 min for NLM Extract, NLM SPE 2 and NLM J. Furthermore, permeated amounts after 24 h were 1.68 ± 0.11, 2.87 ± 0.04 and 3.64 ± 0.22% (w/w), respectively. Phenolic compounds exhibit increase polarity compared to diterpenes, thus, their permeation was delayed in respect to diterpenes, spreading slowly through the skin layers.

Release profile of compounds from developed membranes was evaluated using different mathematical models. Data were analyzed by fitting (SigmaPlot 10.0 software) to different mathematical models.
models (Peppas, Higuchi, First order, Hixon, Baker-Lonsdale and Weibull) through the highest determination coefficient (Table 4). Peppas model showed coefficient for all analyzed compounds. Thus, release data exhibited sigmoid and complex release mechanism once b values were higher than 1 (Papadopoulou et al., 2006; Peppas et al., 2000).

Previous published data of similar studies on pig skin using NLM incorporated with nicotine, demonstrated 10–60% of permeation (w/w) (Pichayakorn et al., 2012b). Once nicotine is highly lipophilic, its absorption is facilitated through skin layers (Cilurzo et al., 2010; Pichayakorn et al., 2012a). Nicotinell® TTS-20 permeation demonstrated similar results in 24 h reaching 15–40% (w/w).

Results of in vitro skin retention (Fig. 7) showed that retained values on epidermis and dermis were higher when compared to stratum corneum for phenolic compounds released from NLM Extract and for diterpenes from NLM SPE 2 and NLM J (p < 0.05).

However, these profiles were different for diterpenes released from NLM Extract. Total retention (epidermis, dermis and stratum corneum) of phenolic compounds for NLM Extract was 10.3% (w/w). Retention of diterpenes was 13.9% (w/w) from NLM Extract, 13.3% (w/w) from NLM SPE 2 and 11.4% (w/w) from NLM J (Fig. 7). Results of diterpenes retention assay from NLM SPE 2 and NLM J did not show statistical differences (p > 0.05). However, statistical significance was found for NLM Extract diterpene retention from NLM J and NLM SPE 2 (p < 0.05). Highest relative retention values found for NLM Extract can be attributed to the higher diterpenes content in this membrane (Table 1). A possible interaction among the compounds or even the higher permeability to water steam of this membrane may facilitate the transport of incorporated active compounds of inner layers of membrane (Akhgari et al., 2006; Pongjanyakul and Puttipipatkhachorn, 2008).

---

**Fig. 3.** SEM images of membranes with 2.000 zoom: (A) NLM, (B) NLM Extract, (C) NLM SPE 2, (D) NLM J.

**Fig. 4.** Water vapor transmission rate profile: mass loss (g) of the membranes per time (h) in the permeability test (n = 3).

**Fig. 5.** Profile of strain per stress of membrane in mechanical strength test (n = 3).
Incorporated active compounds in NLM were retained on skin layers, allowing membranes topical application and providing wound healing and anti-inflammatory properties. C. sylvestris compounds (Albano et al., 2013; Pierri, 2013) with angiogenic activity of NLM compounds (Frade et al., 2004), reducing systemic adverse effects.

**Fig. 6.** Permeation (µg/cm² per h) of diterpenes (n = 6): (A) NLM Extract, (B) NLM SPE 2 and (C) NLM J; and phenolic compounds: (D) NLM Extract.

**Fig. 7.** Obtained values (µg/cm²) of retained phenolic compounds and diterpenes on pig skin layers (mean ± SD; n = 6). Statistical analysis: *NLM Extract diterpene differs from NLM J and NLM SPE 2 p < 0.05. **NLM SPE 2 and NLM J of did not show statistical differences p > 0.05.
Table 4

<table>
<thead>
<tr>
<th>Mathematical model</th>
<th>Parameter</th>
<th>Diterpenes</th>
<th>Phenolic compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NLM Extract</td>
<td>NLM SPE 2</td>
</tr>
<tr>
<td>Baker–Lonsdale</td>
<td>(k)</td>
<td>0.1700</td>
<td>9.5030</td>
</tr>
<tr>
<td></td>
<td>(r^2)</td>
<td>0.9415</td>
<td>0.9605</td>
</tr>
<tr>
<td>First-order</td>
<td>(k_1)</td>
<td>0.0008</td>
<td>0.0014</td>
</tr>
<tr>
<td></td>
<td>(r^2)</td>
<td>0.9082</td>
<td>0.8298</td>
</tr>
<tr>
<td>Higuchi</td>
<td>(k_1)</td>
<td>0.3186</td>
<td>0.5666</td>
</tr>
<tr>
<td></td>
<td>(r^2)</td>
<td>0.9420</td>
<td>0.9780</td>
</tr>
<tr>
<td>Hixon</td>
<td>(k_2)</td>
<td>0.0003</td>
<td>0.0005</td>
</tr>
<tr>
<td></td>
<td>(r^2)</td>
<td>0.9070</td>
<td>0.8229</td>
</tr>
<tr>
<td>Peppas</td>
<td>(k)</td>
<td>0.1975</td>
<td>0.4400</td>
</tr>
<tr>
<td></td>
<td>(r^2)</td>
<td>0.9831</td>
<td>0.9760</td>
</tr>
<tr>
<td></td>
<td>(n)</td>
<td>0.6836</td>
<td>0.5978</td>
</tr>
<tr>
<td>Weibull</td>
<td>(k)</td>
<td>0.1210</td>
<td>0.1410</td>
</tr>
<tr>
<td></td>
<td>(r^2)</td>
<td>0.9841</td>
<td>0.9750</td>
</tr>
<tr>
<td></td>
<td>(b)</td>
<td>30.8623</td>
<td>55.43</td>
</tr>
</tbody>
</table>

For topical systemic, where systemic action is desired permeation values must be high and, as a consequence, retention must be minimal. On the other hand, when a local action is intended, as the evaluated membranes for wound healing and anti-inflammatory actions, the permeation must be avoided and retention values must be high (Sato et al., 2007). Thus, our results demonstrate the potential of developed membranes for the local skin treatment.

In addition to diterpenes NLM Extract contains phenolic compounds and essential oil components (Esteves et al., 2005; Oliveira et al., 2009) which may be related to anti-inflammatory action (Ferreira et al., 2011; Silva et al., 2008). The use of NLM Extract has also technological advantages in production and economy because it does not need fractionation and purification steps, as other evaluated C. sylvestris derivatives.

Authors’ contributions

FAC realized collecting of plants, phytochemical analyses, development and characterization of membranes, permeation and skin retention in vitro analysis, and data interpretation. HSU participated in chromatograph analysis and casearin J purification. FAB and RDH contributed in membrane characterization. MHO and MPDG contributed to permeation and skin retention in vitro assays and interpretation data. AGS participated in the design and coordination of the work and helped to draft the manuscript. All the authors have read contributed to critical reading of the final manuscript and approved its submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by CAPES and Scientific Support and Development Program of School of Pharmaceutical Sciences (Unesp).

References


