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Original Article

# Colombian propolis as starting material for the preparation of nanostructured lipid carriers

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ABSTRACT

The value of propolis is scientifically and commercially measured through the content of biologically active molecules as phenolic compounds and flavonoids; on the other hand, a high percentage of waxes in the propolis composition makes it a substandard beekeeping product. Colombian propolis is characterized by a high content of waxes; however, this drawback turns into an advantage when this material is used for preparing lipid nanocarriers. Accordingly, in this research work, a propolis-extracted material obtained by Randall method is characterized by differential scanning calorimetry, infrared spectroscopy, X-ray diffraction, and $^{1}$H-Nuclear Magnetic Resonance. Then, it is used for obtaining nanostructured lipid carriers by the emulsification–diffusion technique, whose recipe and operating work conditions were established by a Plackett–Burman statistical screening design. The obtained particles exhibit sizes less than 300 nm, polydispersity indices around 0.1, zeta potential values less than ±2 mV, good physical stability and they show to be safe in the *in vitro* irritation test. Thus, Colombian propolis arises as an attractive natural source for obtaining lipid carriers that could be used in pharmaceutical or cosmetic industries for developing innovative products.



## Introduction

Propolis is a resinous hive product, made with exudates, waxes, parts of plants, and metabolites of the bees (Marcucci, 1995; Bankova et al., 2000; de Groot, 2013). Diverse factors such as the surrounding flora and the geographic location of the hive (Salatino et al., 2011), seasonality (Sforcin et al., 2000; Teixeira et al., 2010), illumination, altitude (Toreti et al., 2013), race of bees (Silici and Kutluca, 2005), harvesting methods (Papotti et al., 2012) and activities developed during propolis exploitation (de Groot, 2013) affect its composition.

Usually, propolis is highly valued because of its content of active compounds (Wagh, 2013). However, this concept has changed over time, because other substances present in propolis have been investigated for taking advantage of its composition (Abdulkhani et al., 2015; Chao et al., 2018; Jaganathan et al., 2018; Roh and Kim, 2018). In the special case of the waxes, these can be used as a novel ingredient for lipid carriers (Rassu et al., 2015; Correa González et al., 2016; Kammari et al., 2017; Soleimanian et al., 2018); they are a kind of delivery systems that attract the attention to develop pharmaceutic and cosmetic products because could improve the solubility and bioavailability of lipid-soluble active substances. Particularly, the Nanostructured Lipid Carriers (NLC) are of interest because of their high drug-load capacity, stability, biocompatibility and biodegradability (zur Mühlen et al., 1998; Mehnert and Mäder, 2012; Ganesan and Narayanasamy, 2017; Ghasemiyeh and Mohammadi-Samani, 2018; Gordillo-Galeano and Mora-Huertas, 2018).

In this sense, knowing that some samples of Colombian propolis characterize for having a high content of waxes, this research work investigates their use in the preparation of NLC. Firstly, the propolis lipid material was extracted by the Randall method using ethyl acetate as solvent for obtaining a "propolis-extracted material". Then, NLC from the propolis-extracted material were developed using a statistical experimental design and their size, zeta potential, stability and cytotoxicity were characterized. It is our purpose to generate an added value to the Colombian propolis by developing novelty delivery systems of interest in the pharmaceutical and cosmetic fields.

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**Table 1**
Plackett–Burman screening design to define some variables associated with the formulation and the work conditions for preparing NLC based on propolis-extracted material by using the emulsification–diffusion method.

| Test number | PVA (%) | PLX (%) | CCT (%) | Sesame oil (%) | Emulsification time (min) | Emulsification stirring rate (rpm) |
|---|---|---|---|---|---|---|
| 1 | + | – | + | – | – | – |
| 2 | + | + | – | + | – | – |
| 3 | – | + | + | – | + | – |
| 4 | + | – | + | + | – | + |
| 5 | + | + | – | + | + | – |
| 6 | + | + | + | – | + | + |
| 7 | – | + | + | + | – | + |
| 8 | – | – | + | + | + | – |
| 9 | – | – | – | + | + | + |
| 10 | + | – | – | – | + | + |
| 11 | – | + | – | – | – | + |
| 12 | – | – | – | – | – | – |

## Materials and methods

### *Materials*

Raw propolis was collected from hives of *Apis mellifera* in Confines, Santander, Colombia (6°21′ 23,825″ N, 73° 14′ 30,634″ W, elevation 1498 m) and it was stored at −20 °C. Also, caprylic/capric triglycerides (CCT, Labrafac™ Lipophile WL 1349, gifted by Gattefossé), sesame oil of pharmaceutical quality, poloxamer 188 (PLX, Kolliphor® P188, BASF; gifted by Handler Colombia), polyvinyl alcohol 31–51 kDa with 87–89% of hydrolysis (PVA, Sigma–Aldrich), ethyl acetate (EtAc, Tedia®), Dulbecco's modified eagle's medium (DMEM, Sigma–Aldrich), fetal bovine serum (Eurobia) and penicillin and streptomycin (Life Sciences) were used. Distilled water was used in all experiments (Boeco distiller WS8000).

### Methods

#### *Obtention and characterization of the propolis-extracted material*

*Propolis-extracted material obtention*: The extraction of the propolis was carried out in a solvent extractor Velp® Scientifica SER 148 (solvent: ethyl acetate; immersion time: 60 min; washing time: 50 min; solvent recuperation time: 10 min). The results are reported as the mean of two independent determinations with a 95% confidence interval.

*Characterization of the propolis-extracted material*: Differential Scanning Calorimetry (DSC) was carried out in an inert atmosphere; using around 12 mg of propolis-extracted material (DSC 1 STAR$^e$ System – Mettler Toledo; heating and cooling rates: 5 °C/min; nitrogen flow: 50 ml/min; the samples were heated from −20 °C up to 80 °C, they were maintained 2 min in the last temperature and then, the samples were cooled to −20 °C). Infrared spectroscopy (IR) analysis was performed by diluting the samples with dried KBr (IRAffinity-1 FT-IR Shimadzu; range of scanning: 400–4000 $cm^{-1}$; scanning resolution of 4 $cm^{-1}$; scans number: 45). X-ray diffraction (XRD) pattern was obtained from 2$\theta$ of 10° to 80° (X'PertPRO PANalytical; X-ray source: Cu$K_\alpha$; scan angular speed (2$\theta$/min) of 15°/min; step width: 0.0286°; operating voltage: 40 kV; electrical current: 40 mA). $^1$H-Nuclear Magnetic Resonance ($^1$H-NMR) was carried out at 400 MHz (Bruker Advance 400). Propolis-extracted material (20 mg) was dissolved in deuterated chloroform.

#### *Preparation and characterization of NLC based on propolis-extracted material*

*Preparation of propolis-based NLC*: For this purpose, the emulsification–diffusion technique proposed by Quintanar-Guerrero et al. (2005) was used with some modifications. First, the organic solvent (EtAc) and water were mutually saturated for at least 30 min. Then, two phases were prepared: an organic and an aqueous. For the organic phase, 100 mg of propolis-extracted material was dispersed in 10 ml of water-saturated organic solvent (Ika® C-Mag HS 7; 500 rpm, room temperature); that dispersion was filtered through Whatman No. 3 filter paper and the required quantity of fixed oil (CCT or sesame oil) was added. On the other hand, the aqueous phase was a solution of the stabilizing agent (PLX or PVA) in 40 ml of solvent-saturated water. To prepare the NLC, the organic phase was added to the aqueous phase, and the mix was emulsified at high shear force (Ultra-Turrax® T18 IKA®, 11,200 rpm, 5 min). The obtained emulsion was quickly added to 250 ml of distilled water (Ika® C-Mag HS 7; stirring rate: 500 rpm) and the particles were immediately formed. EtAc and part of the water were retired by vacuum distillation (Heidolph Hei-VAP Precision; 130 rpm, 40 °C) up to a final volume of 80 ml.

The adequate conditions for preparing NLC were established by using a Plackett–Burman statistical screening design of twelve experiments (Table 1). The six selected factors were PLX (2.5% and 5.0%), PVA (2.5% and 5.0%), CCT (0 mg and 10 mg), sesame oil (0 mg and 10 mg), emulsification stirring rate (11,200 rpm and 15,600 rpm), and emulsification time (5 min and 10 min). The response variables were mean particle size, polydispersity index (PDI), and percentage of the main peak of particle size (searching monomodal distributions). The results were processed with the statistical software Statgraphics Centurion V17.1.02.

*Lyophilization of propolis-based NLC*: the solvent EtAc was retired of the NLC with a distillation at reduced pressure (Heidolph Hei-VAP Precision; 130 rpm, 40 °C), the resultant dispersions were disposed in glass containers and they were frozen at -20 °C (Daewoo fridge). The frozen samples were lyophilized during 48 h (Labconco, Freeze drying/Shell Freeze drying).

*Characterization of propolis-based NLC*: Particle size and polydispersity index were measured in samples without preliminary dilution by photon correlation spectroscopy (Zetasizer Nano ZS, Malvern Instruments; 5 measures/sample, 5 runs of 10 s/measure at 25 °C, scattering angle at 173°). Zeta potential was deduced from the electrophoretic mobility measurement by laser Doppler velocimetry (Zetasizer Nano ZS; 5 measures/sample, 10 runs of 10 s/measure at 25 °C); the samples were highly diluted in sodium chloride 1 mM and zeta potential was determined as a function of the pH (ranging between 3 and 9). On the other hand, the stability of the NLC dispersions was evaluated for 30 days, at intervals of 15 days ± 1 day. The samples were stored in transparent glass containers at room temperature (18 °C ± 3 °C) and in an oven at 40 °C ± 1 °C (Jouan IG150). In addition, lyophilized propolis-based NLC were characterized by DSC, IR, XRD, and $^1$H-NMR using

the same work conditions previously described for the extracted material. Complementary, lyophilized propolis-based NLC were observed by Scanning Electron Microscopy (SEM, Tescan Vega 3; acceleration voltage 20 kV; coating: gold/palladium).

Finally, cytotoxicity of lipid nanoparticles was evaluated by Neutral Red Uptake (NRU) assay on BALB/c-3T3 mouse fibroblasts and by (4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT) assay on human keratinocytes. For the NRU assay, cells in exponential growth were cultured in 96-well microtiter plate with supplemented medium (Dulbecco's Modified Eagle's Medium – DMEM, 5% fetal bovine serum, 1% penicillin-streptomycin antibiotics) at a seeding density $1.5 \times 10^4$ cells/well. Cells adhered during 20 h ± 4 h and the medium was removed from the plates after incubation and replaced by one fresh. Subsequently, the NLC were directly added to the cells in concentrations between 50 ppm and 2000 ppm, and they were incubated under standard conditions (37 °C, 65% relative humidity, 5% $CO_2$). After 24 h ± 2 h of incubation, cells were observed by inverted microscopy (Nikon ETS-100) for evaluating possible damages or changes in the cellular morphology. Then, the dispersions of lipid particles were taken off and neutral red dye was added to the cells, that were newly incubated for 3 h ± 0.5 h under the mentioned standard conditions; the cells were washed, and the neutral red dye was extracted. The absorbance of the extracted dye was measured at 550 nm, for determining the percentage of cell survival that was compared with the obtained absorbances from control cell of growth (blank cells). In the study, a nonlinear regression analysis of the percentage of survival *versus* concentration was done for obtaining the values of effective concentrations $EC_{90}$ and $EC_{50}$ (Belcorp, 2012a).

On the other hand, for the MTT test, human keratinocytes in exponential growth were cultured in 96-well microtiter plate at a seeding density $5 \times 10^3$ cells/well and with supplemented medium (DMEM, 5% fetal bovine serum, 1% penicillin-streptomycin antibiotics). Cell adherence was allowed during 20 h ± 4 h and the replacement of medium was done. The NLC dispersions were directly added to the cells in concentrations between 50 ppm and 2000 ppm; and the samples were incubated in standard conditions (37 °C, 65% relative humidity, 5% $CO_2$). After 24 h ± 2 h of incubation, cells were observed by inverted microscopy for evaluating possible damages or changes in the cellular morphology. The treatments were removed and MTT dye was added. Cells were incubated for 2 h ± 0.5 h under the mentioned standard conditions. The formed formazan crystals were extracted with isopropanol and quantified at 575 nm. For estimating $EC_{90}$ and $EC_{50}$ values, the absorbances obtained by the test with lipid particles were related to controls of growth (blank cells) through a nonlinear regression analysis of a curve of percentage of survival *versus* concentration (Belcorp, 2012b).

**Table 2**
Plackett–Burman screening design to develop NLC based on propolis-extracted material: Results for the response (dependent) variables.

| Test | Particle size (nm) | PDI | Main peak (%) |
|---|---|---|---|
| 1 | 244 ± 4 | 0.046 ± 0.034 | 100.0 ± 0.0 |
| 2 | 238 ± 3 | 0.185 ± 0.025 | 98.6 ± 0.8 |
| 3 | 170 ± 3 | 0.064 ± 0.042 | 100.0 ± 0.0 |
| 4 | 134 ± 2 | 0.059 ± 0.011 | 100.0 ± 0.0 |
| 5 | 255 ± 9 | 0.222 ± 0.020 | 98.9 ± 1.6 |
| 6 | 96 ± 2 | 0.161 ± 0.023 | 100.0 ± 0.0 |
| 7 | 127 ± 2 | 0.098 ± 0.036 | 100.0 ± 0.0 |
| 8 | 172 ± 3 | 0.099 ± 0.038 | 100.0 ± 0.0 |
| 9 | 149 ± 2 | 0.135 ± 0.022 | 100.0 ± 0.0 |
| 10 | 131 ± 2 | 0.187 ± 0.017 | 99.3 ± 1.0 |
| 11 | 125 ± 1 | 0.169 ± 0.028 | 99.4 ± 0.9 |
| 12 | 222 ± 4 | 0.087 ± 0.049 | 100.0 ± 0.0 |
| *Statistical parameters of the model* | | | |
| *p*-value | 0.0105 | 0.0215 | 0.0304 |
| $R^2$ (%) | 92.60 | 90.00 | 88.40 |

## Results and discussion

In general terms, raw propolis is composed by 20%–40% of waxes (Woisky and Salatino, 1998; Pietta et al., 2002; Sforcin, 2007; de Groot, 2013; Pujirahayu et al., 2014). However, a significant content of waxes (72%) was found in Colombian propolis, based on what this material is labeled as poor quality. Nevertheless, taking advantage of this drawback, innovative carriers for active compounds could be investigated. As a contribution in this sense, this research work has been focused on NLC, a kind of lipid particles that includes in its composition a mixture of solid and liquid lipids. For this purpose, the extraction and characterization of propolis-extracted material were carried out and then, lipid particles were prepared.

The extraction of the lipid material from propolis enables a more refined raw material to be used for preparing lipid nanoparticles. For this purpose, the Randall extraction method was selected due to its accelerated extraction mechanism (Anderson, 2004) and EtAc was chosen as the extraction solvent because it is the most common organic solvent used for the nanoparticles preparation *via* the emulsification–diffusion technique (Mora-Huertas et al., 2012). The result of the extraction process was 80.8% ± 2.6%, that corresponds to lipid material and perhaps includes other propolis's components also soluble in EtAc.

The extracted material was then used for investigating the more suitable conditions to prepare NLC. In this way, a Plackett–Burman screening design was carried out. The obtained results are compiled in Tables 2 and 3, and in Fig. 1.

In general terms, the Plackett–Burman models adjusted by the Software Statgraphics Centurion V17.1.02 are statistically significant (*p*-values highest than 0.05) and the variations of each

**Table 3**
Plackett-Burman screening design to develop NLC based on propolis-extracted material: Statistical parameters obtained for each variable of interest (independent variables).

| | | PVA (%) | PLX (%) | CCT (%) | Sesame oil (%) | Emulsification time (min) | Emulsification stirring rate (rpm) |
|---|---|---|---|---|---|---|---|
| *Response variables* | | | | | | | |
| Particle size | Sum of squares | 1497.7 | 134.3 | 2614.9 | 609.7 | 1163.1 | 24240.6 |
| | Mean square | 1497.7 | 134.3 | 2614.9 | 609.7 | 1163.1 | 24240.6 |
| | *F* ratio | 3.10 | 0.28 | 5.41 | 1.26 | 2.41 | 50.13 |
| | *p*-value | 0.1387 | 0.6208 | 0.0676 | 0.3125 | 0.1816 | 0.0009[a] |
| PDI | Sum of squares | 0.0036 | 0.0068 | 0.0175 | 0.0006 | 0.0042 | 0.0009 |
| | Mean square | 0.0036 | 0.0068 | 0.0175 | 0.0006 | 0.0042 | 0.0009 |
| | *F* ratio | 4.83 | 9.13 | 23.42 | 0.79 | 5.60 | 1.25 |
| | *p*-value | 0.0793 | 0.0294[a] | 0.0047[a] | 0.4154 | 0.0642 | 0.3136 |
| Main peak | Sum of squares | 0.56 | 0.48 | 1.20 | 0.12 | 0.003 | 0.12 |
| | Mean square | 0.56 | 0.48 | 1.20 | 0.12 | 0.003 | 0.12 |
| | *F* ratio | 8.62 | 7.35 | 18.42 | 1.84 | 0.05 | 1.84 |
| | *p*-value | 0.0324[a] | 0.0423[a] | 0.0078[a] | 0.2333 | 0.8302 | 0.2333 |

[a] Statistically significant effects. *p*-value <0.05.

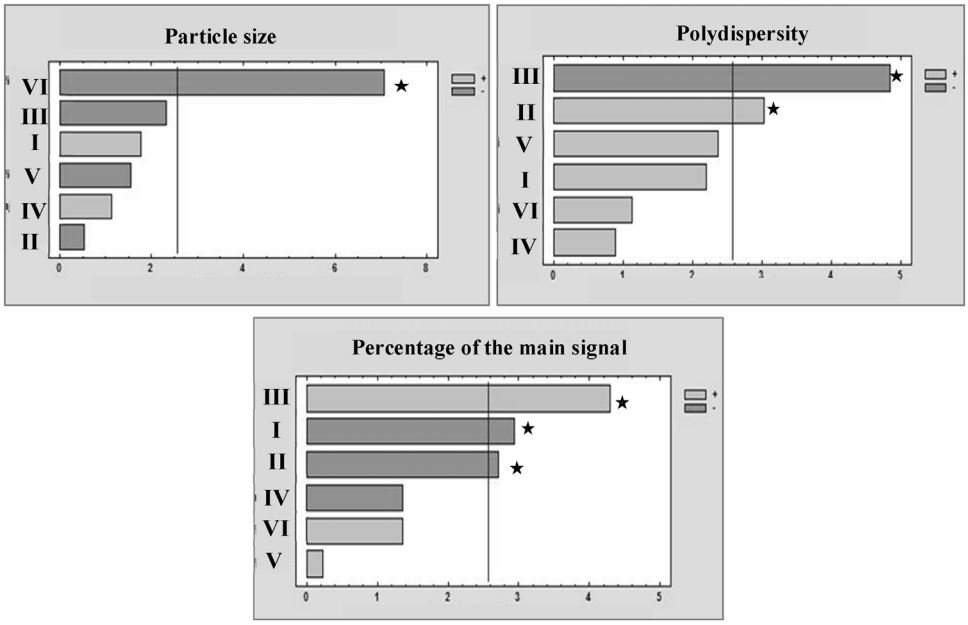


**Fig. 1.** Standardized Pareto charts of the investigated variables by the Plackett–Burman screening design for determining the conditions of NLC preparation. **I**: PVA; **II**: PLX; **III**: CCT; **IV**: sesame oil; **V**: emulsification time; **VI**: emulsification stirring rate. The stars point out the variables with significant effects.

**Table 4**
Interpretation of the results from Plackett–Burman design for formulating NLC based on propolis-extracted material.

| | | PVA conc. | PLX conc. | CCT conc. | Sesame oil conc. | Emulsification time | Emulsification stirring rate |
|---|---|---|---|---|---|---|---|
| Response variables | Particle size | Low | High | High | Low | High | High[a] |
| | PDI | Low | Low[a] | High[a] | Low | Low | Low |
| | Percentage main peak | Low[a] | Low[a] | High[a] | Low | High | High |

[a] Statistically significant effects. $p$-value <0.05.

dependent response are explained by these models in percentages bordering 90%, that is adequate for making decisions on the variables of interest investigated. In this sense, emulsification rate exhibits a significant effect on particle size, PLX and CCT determine the polydispersity of the systems and the concentration of PVA, PLX and CCT governs the percentage of the main signal.

A single integrated view of the results (Table 4) facilitates to make decision on the recommended level of each variable of interest for obtaining particle dispersions exhibiting the smallest size and polydispersity index and the highest percentage of the main peak. Accordingly, the emulsification time does not have an impact on the evaluated characteristics of the particles; therefore, the chosen emulsification time was 5 min. On the other hand, the stirring rate was selected in its low level because the decrease in particle size was not significant in practical terms. Regarding the starting materials, the most adequate option of liquid lipid is CCT, a medium chain triglyceride. Even though the sesame oil is an attractive natural alternative for preparing the particles intended in this research work, this oil should not be incorporated in the formulation probably because of its composition that includes linoleic acid, stearic acid, and linolenic acid (Jannin et al., 2008). All of them are long-chain triglycerides causing a significant increment of the particle size. Finally, the quantity of the PVA and PLX polymers is recommended to be at the low level.

Overall, the selected conditions for obtaining NLC characterized by the lowest size (214 nm ± 3 nm), the lowest PDI (0.09 ± 0.02), and monomodal distributions were 100 mg of propolis-extracted material, 10 mg of CCT, 10 ml of water-saturated EtAc, and 40 ml of solvent-saturated water; that contains 0.5 g of PLX and 0.5 g PVA. The preferred emulsification conditions are 11,200 rpm for 5 min.

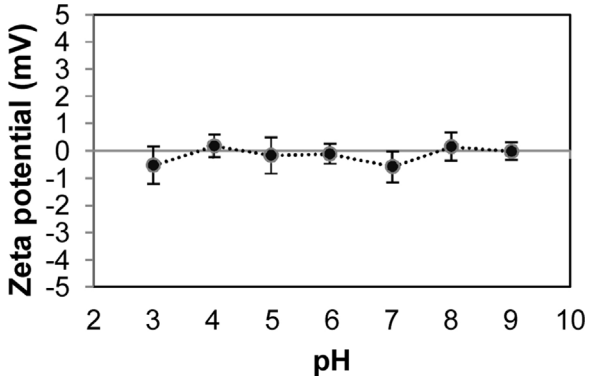


**Fig. 2.** Zeta potential of NLC based on the propolis-extracted material.

The zeta potential of the particles was measured as a function of pH and values near to neutrality were achieved in all cases (Fig. 2). However, a negative zeta potential was expected for instance, because of the free fatty acids present in propolis. An explanation on this respect may be found in the use of non-ionic polymers in the formulation (PVA and PLX), which could shield the electrical charges due to ionized molecules located at the surface of the particle (Napper, 1969; Lourenco et al., 1996). In fact, we have previously reported (Correa González et al., 2016) that propolis-based NLC dispersions exhibit the typical behavior of steric stabilization mechanism with an aggregation critical concentration of 0.7 mM of $Na_2SO_4$.

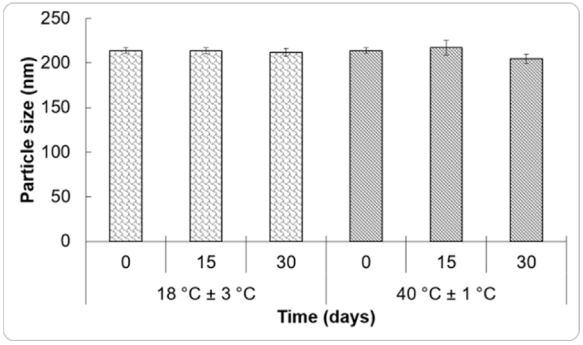


**Fig. 3.** Particle size behavior of NLC based on the propolis-extracted material during the stability test.

In addition, the stability of the propolis-based NLC dispersions was investigated for 30 days at two conditions: 18 °C ± 3 °C and 40 °C ± 1 °C. As shown in Fig. 3, although the particle hydrodynamic diameter increases at the end of the study, this change does not exceed 40 nm; what is not significant for this kind of nanoparticulated systems.

On the other hand, the extracted starting material obtained from propolis was compared against the lyophilized NLC by using DSC analysis. Regarding the starting material, the wide signals obtained could be attributable to the melting and solidification processes (Fig. 4 and Table 5); and the two signals found for the solidification process could be explained by the mix of substances and possible solid–solid transitions for some compounds; for instance, those changes among the crystallographic forms of alkanes could take place (Dorset et al., 1984; Chazhengina et al., 2003; Kameda, 2004). Respect to the lyophilized NLC, their melting interval was displaced to lower temperatures due to the presence of the stabilizing polymers and the CCT. In this sense, it has been demonstrated that the incorporation of CCT modifies the thermal profile of lipid nano-systems (Jenning et al., 2000; Pattarino et al., 2014).

Likewise, infrared spectroscopy allows an initial estimate of the type of functional groups in the extracted material and in the NLC (Fig. 5). In the case of the extracted material, the intense signal between 1700 $cm^{-1}$ and 1750 $cm^{-1}$ could be attributed to carbonyls. The band between 3000 $cm^{-1}$ and 2800 $cm^{-1}$ corresponds

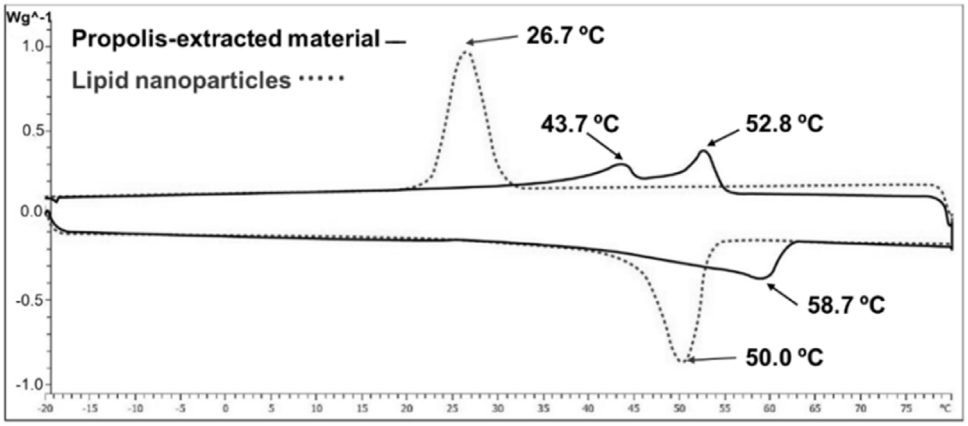


**Fig. 4.** DSC thermogram of EtAc-extracted material (black) and of NLC based on the propolis-extracted material.

**Table 5**
Results of DSC analysis for EtAc-extracted material and for NLC based on propolis-extracted material.

| | Melting range | | | | Solidification range | | | |
|---|---|---|---|---|---|---|---|---|
| | Onset (°C) | Peak (°C) | Enset (°C) | Enthalpy (J/g) | Onset (°C) | Peak (°C) | Enset (°C) | Enthalpy (J/g) |
| EtAc-extracted material | 46.4 | 58.7 | 61.8 | 40.2 | 55.8 | 52.8 | 49.2 | 33.6 |
| Propolis-based lipid particles | 45.3 | 50.0 | 53.2 | 51.6 | 30.4 | 26.7 | 22.9 | −46.3 |

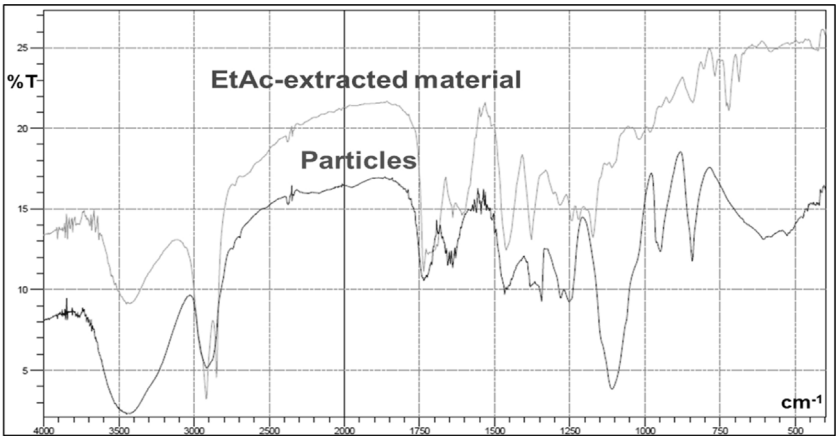


**Fig. 5.** IR spectra (KBr) of EtAc-extracted material (upper) and of NLC based on propolis-extracted material (bottom).

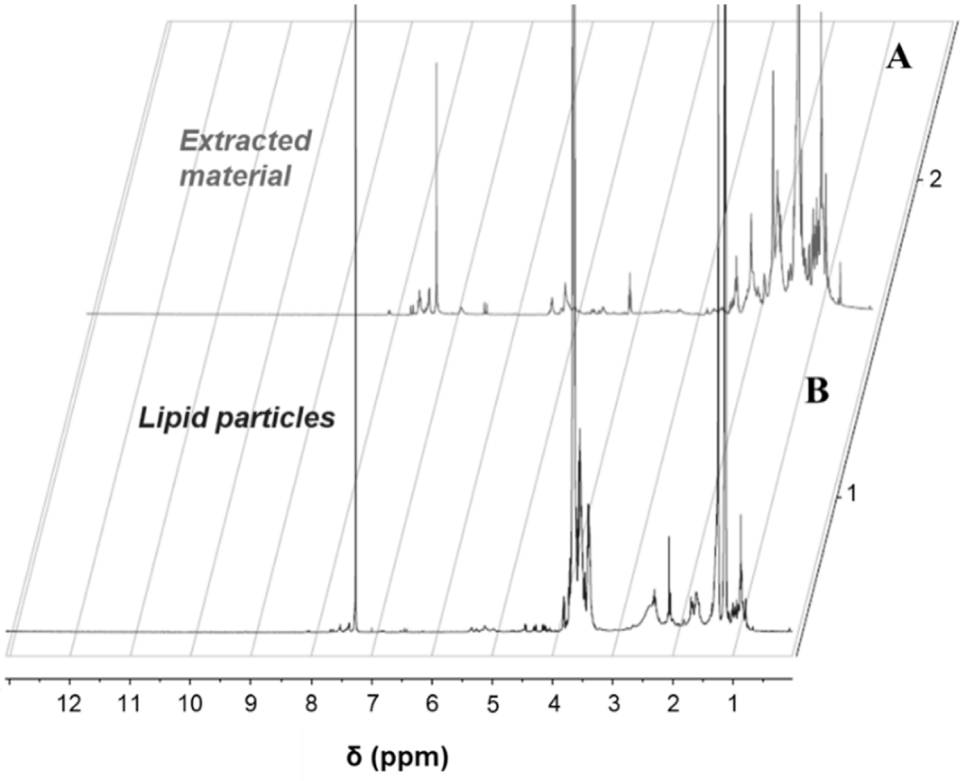


**Fig. 6.** XRD of propolis-extracted material (A) and of NLC based on propolis-extracted material (B).

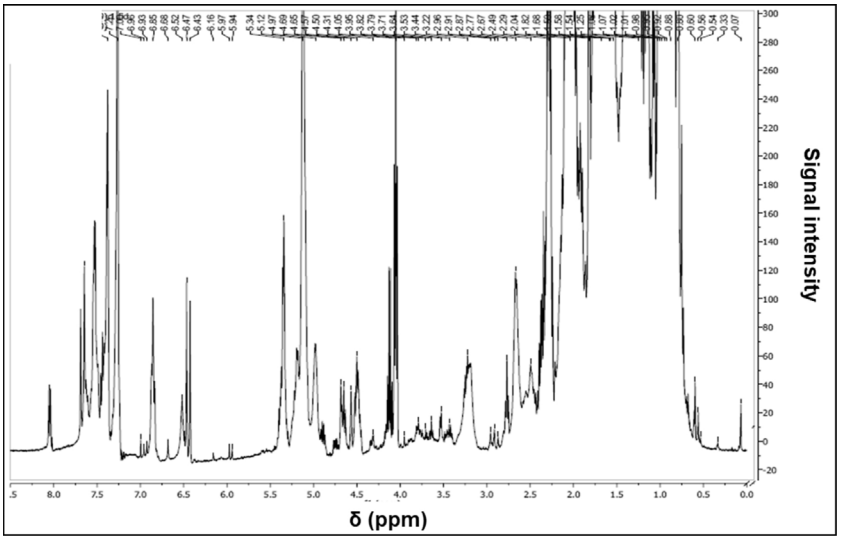


**Fig. 7.** $^{1}$H-NMR spectra (400 MHz, $CDCl_3$) for EtAc-extracted material.

to stretching vibrations of C—H bonds, complemented by vibration of deformation of C—H at 1462 $cm^{-1}$ and hydrocarbon deformations of long chains at 721 $cm^{-1}$ (Silverstein et al., 2005). These results are in line with those previously reported for beeswax (Birshtein and Tul'chinskii, 1977; Zimnicka and Hacura, 2006; Lakshmi et al., 2012; Kajjari et al., 2014). Regarding the NLC, less intense bands than those observed for the extracted material are shown. Thus, the band in the region between 3000 $cm^{-1}$ and 2840 $cm^{-1}$ could be assigned to the stretching vibrations of C—H bonds. In the same way, the band at 1465 $cm^{-1}$ is common for CH scissoring of methylene groups. The weak absorption of C=C stretching of non-conjugated alkenes appears at 1640 $cm^{-1}$. In addition, absorption at 3700 $cm^{-1}$ and 3200 $cm^{-1}$ is related with O—H stretching vibrations and there is a sharp signal at 1735 $cm^{-1}$, attributable perhaps to carboxyl groups belonging to esters. The signals of the stabilizing polymers are represented by the bands at 1110 $cm^{-1}$, 840 $cm^{-1}$, and 948 $cm^{-1}$; corresponding to C—O bonds (Silverstein et al., 2005).

When propolis-extracted material and nanoparticles were analyzed by XRD, in both cases crystalline regions were evidenced (Fig. 6), but their performance is mainly amorphous. Nevertheless, the observed behaviors are different. The typical $2\theta$ angle at 16.8° of extracted material disappeared in the diffractograms of NLC. Probably, this is due to the crystalline behavior of PVA (Bunn, 1948; Tretinnikov and Zagorskaya, 2012) and PLX (Ramadhani

**Table 6**
Results of the cytotoxicity tests for propolis-based NLC.

| Cytotoxicity in BALB/c-3T3 mouse fibroblasts (red neutral uptake) | | | |
|---|---|---|---|
| Used concentration | HTD (ppm) | $EC_{90}$ (ppm) | Final concept |
| Eight different concentrations between 50 ppm and 2000 ppm | >2000 | >2000 | Minimum irritant |

| Cytotoxicity in human keratinocytes (metabolic reduction of MTT) | | |
|---|---|---|
| Used concentration | $EC_{50}$ (ppm) | $EC_{90}$ (ppm) |
| Eight different concentrations between 50 ppm and 2000 ppm | 839.5 | 324.3 |

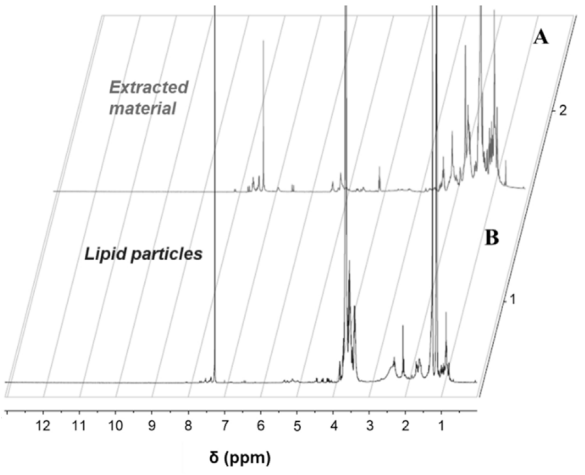


**Fig. 8.** Comparison of $^1$H-NMR spectra (400 MHz, $CDCl_3$) for EtAc-extracted material (A) and NLC based on propolis-extracted material (B).

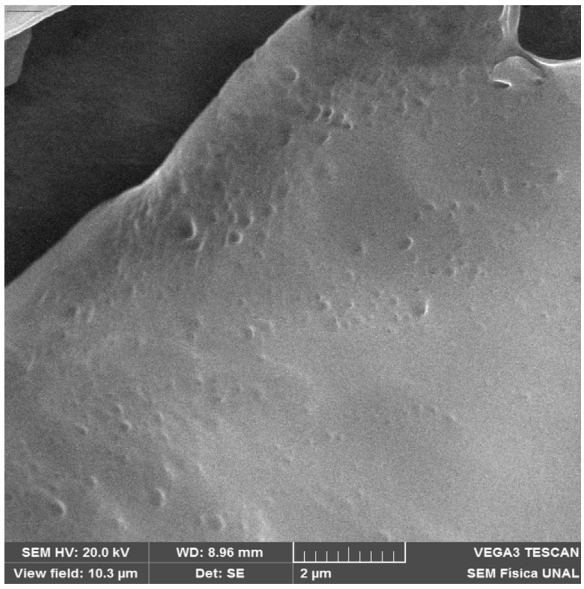


**Fig. 9.** Microphotography of lyophilized NLC based on propolis-extracted material inside of a polymeric matrix.

et al., 2014) that influences the crystalline structure of the particles (Malaki Nik et al., 2012; Malamatari et al., 2015).

$^1$H-NMR has been used for propolis characterization (Watson et al., 2006; Cuesta-Rubio et al., 2007; Bertelli et al., 2012), and therefore, this technique was used to characterize the propolis-extracted material and NLC. For the starting material, the signals found between 0.5 ppm and 2.0 ppm are coherent with the presence of hydrocarbon material (Fig. 7). The presence of hydrogens of alkenes, in the ranges of 4.5 ppm–6.5 ppm (R—C═C—H), and aromatic protons, identified by the signals between 7 ppm and 8 ppm, suggest the existence of phenolic compounds in the extracted materials.

On the other hand, the $^1$H-NMR spectrum for the NLC (Fig. 8) shows a predominant acyclic aliphatic region for the chemical shift ($\delta_H$) between 0.5 ppm and 2.0 ppm; and protons of aromatic compounds or near to heteroatoms are present (6.0 ppm–9.0 ppm), as in the extracted material. The peaks at 3.5 ppm, 3.63 ppm, and 3.8 ppm are intense. They could be attributable to hydrogens adjacent to oxygen, as those of ethers, esters, or hydroxyls; that are present in the structure of PLX and PVA. Concerning to PVA, the reported research works of Petit and Zhu (1996) in deuterated aqueous solutions of PVA evidenced that there is a wide signal between 3.9 ppm and 4.1 ppm of C—H near to oxygen, while the $\delta_H$ near to 3.7 ppm corresponds to the hydroxyls. Equally, for PLX, it has been reported a signal around 3.7 ppm for the groups C—$H_2$ and CH, present in the three monomers of the polymer (Forshed et al., 2005). Complementarily, using SEM, the lyophilized propolis-based NLC with gold-palladium coating were observed embedded in its surrounding polymeric matrix (Fig. 9), exhibiting rounded or lentils shapes with sizes in the nanometric range.

Finally, considering the possible use of the propolis-based NLC for developing delivery systems of interest in pharmaceutics and cosmetics, the *in vitro* cytotoxicity of lipid nanoparticles was investigated. The results reveal that the NLC investigated do not have a toxic effect on BALB/c-3T3 mouse fibroblasts (Table 6), in concordance with the parameters previously reported (Belcorp, 2012a,b), where a "minimum irritant" is the substance whose highest tolerated dose (HTD) is higher than 200 ppm and its $EC_{90}$ is more than 500 ppm. The development of both NRU assay and MTT assay in this research work is complementary; they are simultaneously used because of their different basic principles (Doktorovova et al., 2014). Thereby, MTT is converted by the mitochondrial succinate-dehydrogenase enzyme to formazan, in metabolically active cells (Mosmann, 1983); whereas neutral red dye is accumulated in lysosomes of living cells (Repetto et al., 2008).

## Conclusions

The Colombian propolis obtained from hives of *Apis mellifera* in Confines (Santander, Colombia) can be successfully used in the preparation of NLC *via* the emulsification–diffusion method. The obtained particulate systems exhibit unimodal distributions and achieved a nanometric range with values of polydispersity lower than 0.1. Differences were detected when the propolis-extracted material was compared with NLC using DSC, IR, XRD, and $^1$H-NMR. Likewise, the evaluation of cytotoxicity *in vitro* of lipid nanoparticles allowed to classify them as a minimum irritant.

## Conflicts of interest

The authors declare no conflicts of interest.

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## References

Abdulkhani, A., Hosseinzadeh, J., Ashori, A., Esmaeeli, H., 2015. Evaluation of the antibacterial activity of cellulose nanofibers/polylactic acid composites coated with ethanolic extract of propolis. Polym. Composite 38, 13–19.

Anderson, S., 2004. Soxtec: its principles and applications. In: Luthria, D. (Ed.), Oil Extraction and Analysis: Critical Issues and Competitive Studies. AOCS Press, Champaign, pp. 11–12.

Bankova, V.S., de Castro, S.L., Marcucci, M.C., 2000. Propolis: recent advances in chemistry and plant origin. Apidologie 31, 3–15.

Belcorp, 2012a. In vitro Safety screening to predict ocular irritation of cosmetic formulations. Protocol #3: neutral red uptake cytotoxicity assay in mammalian cells (fibroblast). Tocancipá.

Belcorp, 2012b. In vitro safety screening to predict ocular irritation of cosmetic formulations. Protocol #4: in vitro ocular irritation test using the EpiOcular™ model. Tocancipá.

Bertelli, D., Papotti, G., Bortolotti, L., Marcazzan, G.L., Plessi, M., 2012. $^{1}$H-NMR simultaneous identification of health-relevant compounds in propolis extracts. Phytochem. Anal. 23, 260–266.

Birshtein, V.Y., Tul'chinskii, V.M., 1977. Determination of beeswax and some impurities by IR spectroscopy. Chem. Nat. Compd. 13, 232–235.

Bunn, C.W., 1948. Crystal structure of polyvinyl alcohol. Nature 161, 929–930.

Chao, C.Y., Mani, M.P., Jaganathan, S.K., 2018. Engineering electrospun multicomponent polyurethane scaffolding platform comprising grapeseed oil and honey/propolis for bone tissue regeneration. PLoS ONE 13, 1–17.

Chazhengina, S.Y., Kotelnikova, E.N., Filippova, I.V., Filatov, S.K., 2003. Phase transitions of n-alkanes as rotator crystals. J. Mol. Struct. 647, 243–257.

Correa González, Y.X., Paillié Jiménez, D., Valenzuela Correa, A.L., Rojas Cardozo, M.A., Mora Huertas, C.E., 2016. Nanopartículas lipídicas a base de propóleos como transportadores de activos de interés cosmético: desarrollo, caracterización y evaluación de seguridad. Rev. Colomb. Cienc. Quím. Farm. 45, 147–168.

Cuesta-Rubio, O., Piccinelli, A.L., Fernandez, M.C., Hernández, I.M., Rosado, A., Rastrelli, L., 2007. Chemical characterization of Cuban propolis by HPLC–PDA, HPLC–MS, and NMR: the brown, red, and yellow Cuban varieties of propolis. J. Agric. Food Chem. 55, 7502–7509.

de Groot, A.C., 2013. Propolis: a review of properties, applications, chemical composition, contact allergy, and other adverse effects. Dermatitis 24, 263–282.

Doktorovova, S., Souto, E.B., Silva, A.M., 2014. Nanotoxicology applied to solid lipid nanoparticles and nanostructured lipid carriers – a systematic review of in vitro data. Eur. J. Pharm. Biopharm. 87, 1–18.

Dorset, D.L., Moss, B., Wittmann, J.C., Lotz, B., 1984. The pre-melt phase of n-alkanes: crystallographic evidence for a kinked chain structure. Proc. Natl. Acad. Sci. U. S. A. 81, 1913–1917.

Forshed, J., Erlandsson, B., Jacobsson, S.P., 2005. Quantification of aldehyde impurities in poloxamer by $^{1}$H NMR spectrometry. Anal. Chim. Acta 552, 160–165.

Ganesan, P., Narayanasamy, D., 2017. Lipid nanoparticles: different preparation techniques, characterization, hurdles, and strategies for the production of solid lipid nanoparticles and nanostructured lipid carriers for oral drug delivery. Sustain. Chem. Pharm. 6, 37–56.

Ghasemiyeh, P., Mohammadi-Samani, S., 2018. Solid lipid nanoparticles and nanostructured lipid carriers as novel drug delivery systems: applications, advantages and disadvantages. Res. Pharm. Sci. 13, 288–303.

Gordillo-Galeano, A., Mora-Huertas, C.E., 2018. Solid lipid nanoparticles and nanostructured lipid carriers: a review emphasizing on particle structure and drug release. Eur. J. Pharm. Biopharm. 133, 285–308.

Jaganathan, S.K., Mani, M.P., Ismail, A.F., Prabhakaran, P., Nageswaran, G., 2018. Tailor-made multicomponent electrospun polyurethane nanofibrous composite scaffold comprising olive oil, honey, and propolis for bone tissue engineering. Polym. Composite 40, 1–12.

Jannin, V., Musakhanian, J., Marchaud, D., 2008. Approaches for the development of solid and semi-solid lipid-based formulations. Adv. Drug Deliv. Rev. 60, 734–746.

Jenning, V., Thünemann, A.F., Gohla, S.H., 2000. Characterisation of a novel solid lipid nanoparticle carrier system based on binary mixtures of liquid and solid lipids. Int. J. Pharm. 199, 167–177.

Kajjari, P.B., Manjeshwar, L.S., Aminabhavi, T.M., 2014. Novel blend microspheres of cellulose triacetate and bee wax for the controlled release of nateglinide. J. Ind. Eng. Chem. 20, 397–404.

Kammari, R., Das, N.G., Das, S.K., 2017. Nanoparticulate systems for therapeutic and diagnostic applications. In: Mitra, A., Cholkar, K., Mandal, A. (Eds.), Emerging Nanotechnologies for Diagnostics, Drug Delivery, and Medical Devices. Elsevier, Indianapolis, pp. 105–144.

Kameda, T., 2004. Molecular structure of crude beeswax studied by solid-state $^{13}$C NMR. J. Insect Sci. 4, 1–5.

Lakshmi, M., Ahmad, A., Krishna, T.R., Bellubbi, B.S., 2012. A study on composition and structure of beeswax. J. Chem. Biol. Phys. Sci. 2, 2036–2043.

Lourenco, C., Teixeira, M., Simões, S., Gaspar, R., 1996. Steric stabilization of nanoparticles: size and surface properties. Int. J. Pharm. 138, 1–12.

Malaki Nik, A., Langmaid, S., Wright, A.J., 2012. Nonionic surfactant and interfacial structure impact crystallinity and stability of β-carotene loaded lipid nanodispersions. J. Agric. Food Chem. 60, 4126–4135.

Malamatari, M., Somavarapu, S., Bloxham, M., Buckton, G., 2015. Nanoparticle agglomerates of indomethacin: the role of poloxamers and matrix former on their dissolution and aerosolisation efficiency. Int. J. Pharm., 516–526.

Marcucci, M.C., 1995. Propolis: chemical composition, biological properties and therapeutic activity. Apidologie 26, 83–99.

Mehnert, D., Mäder, K., 2012. Solid lipid nanoparticles. Producción, characterization and applications. Adv. Drug Deliv. Rev. 64, 83–101.

Mora-Huertas, C.E., Garrigues, O., Fessi, H., Elaissari, A., 2012. Nanocapsules prepared via nanoprecipitation and emulsification–diffusion methods: comparative study. Eur. J. Pharm. Biopharm. 80, 235–239.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Napper, D.H., 1969. The steric stabilization of hydrosols by nonionic macromolecules. J. Colloid Interface Sci. 29, 168–170.

Papotti, G., Bertelli, D., Bortolotti, L., Plessi, M., 2012. Chemical and functional characterization of Italian propolis obtained by different harvesting methods. J. Agric. Food Chem. 60, 2852–2862.

Pattarino, F., Bettini, R., Foglio Bonda, A., Della Bella, A., Giovannelli, L., 2014. Polymorphism and kinetic behavior of binary mixtures of triglycerides. Int. J. Pharm. 473, 87–94.

Petit, J.-M., Zhu, X.X., 1996. 1H and 13C NMR study on local dynamics of poly(vinyl alcohol) in aqueous solutions. Macromolecules 29, 2075–2081.

Pietta, P.G., Gardana, C., Pietta, A.M., 2002. Analytical methods for quality control of propolis. Fitoterapia 73 (Suppl. 1), S7–S20.

Pujirahayu, N., Ritonga, H., Uslinawaty, Z., 2014. Properties and flavonoids content in propolis of some extraction method of raw propolis. Int. J. Pharm. Pharm. Sci. 6, 338–340.

Quintanar-Guerrero, D., Tamayo-Esquivel, D., Ganem-Quintanar, A., Allémann, E., Doelker, E., 2005. Adaptation and optimization of the emulsification–diffusion technique to prepare lipidic nanospheres. Eur. J. Pharm. Sci. 26, 211–218.

Ramadhani, N., Shabir, M., McConville, C., 2014. Preparation and characterisation of Kolliphor® P 188 and P 237 solid dispersion oral tablets containing the poorly water soluble drug disulfiram. Int. J. Pharm. 475, 514–522.

Rassu, G., Cossu, M., Langasco, R., Carta, A., Cavalli, R., Giunchedi, P., Gavini, E., 2015. Propolis as lipid bioactive nano-carrier for topical nasal drug delivery. Colloids Surf. B: Biointerfaces 136, 908–917.

Repetto, G., del Peso, A., Zurita, J.L., 2008. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat. Protoc. 3, 1125–1131.

Roh, J., Kim, K.R., 2018. Antimicrobial activity of Korean propolis extracts on oral pathogenic microorganisms. J. Dent. Hyg. Sci. 18, 18–23.

Salatino, A., Fernandes-Silva, C.C., Righi, A.A., Salatino, M.L.F., 2011. Propolis research and the chemistry of plant products. Nat. Prod. Rep. 28, 925–936.

Sforcin, J.M., 2007. Propolis and the immune system: a review. J. Ethnopharmacol. 113, 1–14.

Sforcin, J.M., Fernandes Jr., A., Lopes, C.A.M., Bankova, V., Funari, S.R.C., 2000. Seasonal effect on Brazilian propolis antibacterial activity. J. Ethnopharmacol. 73, 243–249.

Silici, S., Kutluca, S., 2005. Chemical composition and antibacterial activity of propolis collected by three different races of honeybees in the same region. J. Ethnopharmacol. 99, 69–73.

Silverstein, R.M., Webster, F.X., Kiemble, D.J., 2005. Spectrometric Identification of Organic Compounds, 7th ed. John Wiley & Sons, INC, Hoboken, New Jersey.

Soleimanian, Y., Hossein Goli, S.A., Varshosaz, J., Maestrelli, F., 2018. Propolis wax nanostructured lipid carrier for delivery of β sitosterol: effect of formulation variables on physicochemical properties. Food Chem. 260, 97–105.

Teixeira, É.W., Message, D., Negri, G., Salatino, A., Stringheta, P.C., 2010. Seasonal variation, chemical composition and antioxidant activity of Brazilian propolis samples. Evid.-Based Complement. Altern. Med. 7, 307–315.

Toreti, V.C., Sato, H.H., Pastore, G.M., Park, Y.K., 2013. Recent progress of propolis for its biological and chemical compositions and its botanical origin. Evid.-Based. Complement. Alternat. Med. 2013, 1–13.

Tretinnikov, O.N., Zagorskaya, S.A., 2012. Determination of the degree of crystallinity in solid–solid equilibria. J. Appl. Spectrosc. 79, 521–526.

Wagh, V.D., 2013. Propolis: a wonder bees product and its pharmacological potentials. Adv. Pharmacol. Sci. 2013, 1–11.

Watson, D.G., Peyfoon, E., Zheng, L., Lu, D., Seidel, V., Jonhston, B., Parkinson, J.A., Fearnley, J., 2006. Application of principal components analysis to $^{1}$H-NMR data obtained from propolis samples of different geographical origin. Phytochem. Anal. 17, 323–331.

Woisky, R.G., Salatino, A., 1998. Analysis of propolis: some parameters and procedures for chemical quality control. J. Apicult. Res. 37, 99–105.

Zimnicka, B., Hacura, A., 2006. An investigation of molecular structure and dynamics of crude beeswax by vibrational spectroscopy. Polish J. Environ. Stud. 15, 112–114.

zur Mühlen, A., Schwarz, C., Mehnert, W., 1998. Solid lipid nanoparticles (SLN) for controlled drug delivery-drug release and release mechanism. Eur. J. Pharm. Biopharm. 45, 149–155.