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Preparation and characterisation of organic UV filters based on combined PHB/liposomes with natural phenolic compounds

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ABSTRACT

In this study preparation and characterization of new UV-protecting systems based on liposomes/polyhydroxybutyrate (PHB) with encapsulated coffee extracts are presented. Green and roasted coffee extracts with high phenolics content, high antioxidant activity and sun protection factor (SPF) value 40–50 were used as model organic UV filters and encapsulated into liposomes and PHB-liposomes. Particle size and colloid stability was observed by dynamic light scattering and zeta-potential. Toxicity of particles was tested by MTT and LDH assay on HaCaT cell line. All prepared samples showed moderate or high encapsulation efficiency. Addition of PHB up to 50 % of lecithin led to increased size and stability. As optimal addition of 20 % PHB into liposome particles was found leading to optimum size and processing of particles, to high UV-protective effect as well as to increased colloid stability and SPF value during long-term storage. Significant differences in cell viability were found in cytotoxicity studies after exposure of keratinocytes to liposomes with different PHB content. Newly fabricated PHB-liposome particles with coffee extract were not found as toxic for HaCaT cells and in LDH test up to 12 %. These particles can act as active carriers for organic sunscreen components in combination with UV-protective effect of PHB.

1. Introduction

The Sun is a source of electromagnetic radiation composed from several portions, such as infrared, visible, and ultraviolet light. Sunlight is filtered through atmosphere and the Earth surface is reached by approximately 50 % of infrared, 45 % visible and 5% of ultraviolet light component (UV; Lautenschlager et al., 2007). UV radiation is commonly separated in UVA (320 − 400 nm), UVB (290 − 320 nm) and UVC (200 − 290 nm) components. UVC is absorbed by the ozone layer and practically does not reach the Earth surface, while UVB radiation is highly energetic and causes skin damage (Rezende et al., 2014). UVA rays are less energetic and substantially less harmfull, nevertheless, they penetrate into the dermis and can negtively affect skin structural components (Lim and Cooper, 1999).

The ultraviolet component of sunlight can exhibits positive as well as negative health effects. It is necessary for beneficial transformation of vitamin D_2 to D_3 and, simultaneously, it can act as a mutagen. Long-term exposure to UV light is known to be associated with serious skin damage, such as skin cancer, skin aging, inflammation and immune suppression, as well as with some eye diseases. Short-term overexposure is the cause of sunburn, erythema and solar retinopathy (Heenen et al., 2001). UV rays are the only listed carcinogens that are known to have health benefits, and, thus, a balance between the risks of having too much sunlight or too little should be found (Burnett and Wang, 2011).

The excess radiation promotes interaction of skin chromophores with molecular oxygen, resulting in oxidative stress. Increased formation of reactive oxygen species (ROS) causes increased lipid peroxidation, DNA damage, enzyme inactivation, decreased levels of antioxidants, and consequently, pathologic changes of skin (Lautenschlager et al., 2007). Acute and chronic negative effects of sunlight were classified. To the acute effects belong sunburn, erythema and drug- photosensitivity, while to the chronic effects belong photoaging, immunosuppression and photocarcinogenesis (Rezende et al., 2014).

Skin has several protection mechanisms how to protect itself from negative effects of UV radiation. Nevertheless, medical organizations recommend the use of sunscreens in the prevention skin cancer (Lim and Cooper, 1999). Sunscreens are cosmetic products with UV filters and antioxidants, which can absorb, reflect and scatter UV light reaching the skin surface (Moyal, 2012). Sunscreens can be classified into physical and chemical systems. Physical sunscreens such as zinc oxide (ZnO), titanium dioxide (TiO_2) and others stay on the skin surface

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and mainly deflect the sunlight, while chemical sunscreens (called also UV organic filters) can absorb, reflect and scatter the UV light at the same time with the ratio of about 90 % of absorption and 10 % of scattering (Catelan et al., 2019).

Organic filters are mostly aromatic compounds with conjugated system of double bonds and some carbonyl groups. These structural components are called chromophores and are responsible for the absorption of particular part of UV spectrum. After absorption UV light these molecules release lower energy rays without some degradation (Shaath, 2007).

Inorganic and organic suncsreens and their mixtures protect the skin against UVA and UVB radiation. They must comply some legislation criteria, for example stability under the UV irradiation and heat for several hours, low irritation potential, water insolubility and many others. Nowadays, adverse effects of the TiO_2 and ZnO nanoparticles on viable skin cells are broadly studied (FDA, 2007) and, thus, organic filtres in some stabilized form are preferred by producers as well as consumers (Catelan et al., 2019).

Sunscreens are commonly rated and labeled with a sun protection factor (SPF). This parameter can be measured *in vitro* or *in vivo* to evaluate the portion of UV rays producing skin sunburn damage. *in vivo* measurement needs volunteers, to which sunscreen is applied to the skin and time before sunburn manifestation under UV lamp is measured (Rezende et al., 2014). The *in vitro* method is based on spectrophotometry in UV part of spectrum. This method can be considered as a rapid, easy and safe preliminary test (Mansur et al., 1986) and and is widely used for identification of substances with potential of UV filter. In this test, the transmittance/absorbance of the sunscreen is recorded in the part of wavelenghts corresponding to UVB–UVA range (290–400 nm) and evaluated using a table of erythemal action spectrum and the standard intensity spectrum of sunlight (Mansur et al., 1986). Results of this type of *in vitro* test respond very well to results of *in vivo* testing.

It is widely recognized that for final sunscreen composition are crucial not only the structure and properties of the filter molecules, but also additional substances such as vehicula or carriers (Puglia et al., 2014). Commonly used application forms (such as emulsion, gels and oils) exhibit some limitations, above all higher percutaneous absorption, washability and low stability. Thus, some carriers of a new type have been proposed, such as nanoemulsions, liposomes and nanoparticles (Morganti, 2010). Sunscreens based on nanoparticles with encapsulated organic UV filters ensure basic demands on the quality of such cosmetic forms – excellent adhesion on the skin surface and, simultaneously, low penetration to the skin and ability to protect the skin against both UV-induced damage and free radicals (Deng et al., 2015).

Liposomes are membrane-based vesicles widely used as delivery systems for drugs and cosmetics. They are fully biocompatible and able to house both water-soluble and lipid-soluble compounds. Tne unadvantage of liposomes is decreased stability and gradual release of encapsulated molecules during long-term storage. One of promissing approaches to enhance liposomes stability is combination with other components, particularly with biodegradable polymeric compounds. One of material suitable to influence positively stability of liposomes and control release of active components is poly(3-hydroxybutyrate) (PHB). This bacterial storage polymer exhibits various promising attributes, such as biocompatibility, biodegradability and, moreover, UV absorption (Slaninova et al., 2018). Combined PHB-liposomes can influence adhesion of carrier particles to the skin and control release of active agents over a period of time (Bokrova et al., 2019).

A long time ago people have used a variety of plant products to protect the skin from harmful effect of sun radiation. Some of these products are still used as active components of sunscreens today (Choquenet et al., 2008). Plant extracts can increase the SPF value of inorganic and organic filters by synergistic activity. Plant extracts and chemical substances isolated from some plants can protect the skin by several mechanisms: as organic UV filters, antioxidants and anti-inflammatory compounds. These effects lead to minimizing the skin damage caused by UV radiation (Choquenet et al., 2008). In this sense, plant species containing high level of phenolic compounds stand out (Djeridane et al., 2006). Derivatives of caffeic acids, resveratrol, quercetin, ferulic acids, apigenin, genistein and tannins (and many others) belong to the group of natural compounds with high protection activity against solar radiation contributing to enhancement of SPF value (Jaradata et al., 2018). One of plants with extremely high content of such natural phenolic substances is coffee (Hečimovič et al., 2011).

Coffee beans of two different species of *Coffea* genus can be found on tne market: *Coffea arabica* and *Coffea canephora* syn. *Coffea robusta*. Coffee presents a rich source of many biologically active compounds, such as nicotinic acid, trigonelline, quinolinic acid, tannic acid, pyrogallic acid and especially caffeine. Further, coffee provides a high content of phenolic acids of the hydroxycinnamic acids family (caffeic, chlorogenic, coumaric, ferulic and sinapic acids; Hečimovič et al., 2011). Coffee is also the major source of chlorogenic acids and contributes significantly to the total polyphenol intake in human diet. In some studies high *in vitro* antioxidant activity of coffee was reported by Moreira et al. (2005) and Hečimovič et al. (2011).

The quality of coffee is generally related to the species and chemical composition of green beans, to post-harvest processing conditions influencing substantially the chemical composition of the roasted beans. In this study extracts from different types of processed coffee will be tested as model photoprotective agent. Coffee extracts will be encapsulated into combined liposome/polyhydroxybutyrate particles. It can be suggested that these particles could serve as carriers for active compounds in topical sunscreens with additional UV-scattering effect of PHB (Bokrova et al., 2019). To author´s best knowledge such complex organic sunscreen system was not proposed yet. Therefore, in this study PHB/liposome particles will be used to development of a new UV protective system by using encapsulation of extracts from *Coffea arabica* as source of antioxidants.

2. Material and methods

2.1. Extraction and characterization of active compounds from Coffea arabica

Active compounds from *Coffea arabica* were extracted by using mixture of deionized water and UV–vis ethanol (20, 40, 60, 80 and 96 %). For extraction 1 g of coffee beans sample was milled in coffee mill and steered with 10 mL of extraction solution for 24 h at laboratory temperature.

2.1.1. Total phenolics

Determination of the total phenolics content (TPC) present in the water and ethanolic extract samples was performed using modified Folin-Ciocalteu method (Ramon-Goncalves et al., 2019). Folin-Ciocalteau reagent was diluted 1:9 with deionized water. Then, 9 mL of diluted reagent were added to 1 mL od distilled water and 50 µL of sample. This solution was mixed by vortex, after 5 min 1 mL of saturated sodium carbonate solution was added and repeatly mixed. Absorption was measured after 15 min at 750 nm. For calibration curve gallic acid solution in the range of 0.1–0.7 mg mL^{-1} was used as standard. Values are represented as mean ± SD of three replicate determinations.

2.1.2. Total flavonoids

The total flavonoids content (TFC) of the extracts was determined using a slightly modified spectrophotometric method (Meda et al., 2005). Sample of 0.5 mL was pipetted into test tube and 1 mL of deionized water and 0.2 mL of 5% $NaNO_2$ solution were added. Mixture was stirred and after 5 min 0.2 mL of 10 % $AlCl_3$ was added and stirred. Additionally, 1.5 mL of 1 M NaOH and 1 mL of deionized water was added. After 10 min absorption was measured at 510 nm. Water was

used as blank. Calibration curve was done with katechin in the concentration range of 0.05–0.37 mg mL^{-1}. Katechin was dissolved in ethanol. Concentrations of flavonoids were calculated using calibration equation. Values are represented as mean ± SD of three replicate determinations.

2.1.3. Antioxidant activity

For evaluation of antioxidant activity Trolox equivalent assay with ABTS radical was performed (Re et al., 1999). ABTS radical was prepared by mixing 7 mM ABTS (2,2´–azino–bis(3–ethylbenzothiazoline–6–sulfuric acid) diammonium salt) (Sigma–Aldrich), potassium persulfate (Sigma–Aldrich), with 2.45 mM potassium persulphate in deionized water and let in dark for 12 h. Before using $ABTS^{\cdot+}$ was diluted with UV–vis ethanol for absorption A = 0.70 at 734 nm with ethanol as blank. For measurement of antioxidant activity 1 mL of $ABTS^{\cdot+}$ and 10 µL of sample was added into eppendorf test tube and decrease of absorption was measured after 10 min. Calibration curve was made by Trolox in the range of concentration 50–400 µg·mL^{-1}. Values are represented as mean ± SD of three replicate determinations.

2.1.4. SPF measurement

Prepared extracts were diluted with UV–vis ethanol to concentration of 200 µg $mL^{-1}$and absorption spectra were measured at wavelengths in the range of 290–320 nm. UV–vis ethanol was used as blank. Calculation of SPF was done according to following Mansur Eq. (1), where CF is correlation factor (10), EE (λ) is erotogenic effect at certain wavelength and Abs (λ) is absorption of sample according to wavelength. Values of EE·I are constants introduced in Table 1 (Sayre et al., 1979). Values are represented as mean ± SD of three replicate determinations.

$$SPF = CF \cdot \sum_{290}^{320} EE(\lambda) \cdot I(\lambda) \cdot Abs(\lambda) \quad (1)$$

Values of constants to calculate SPF by Mansur equation

λ [nm]	290	295	300	305	310	315	320
EE·I	0.015 0	0.081 7	0.287 4	0.327 8	0.186 4	0.083 7	0.018 0

2.2. Particle preparation and characterization

Encapsulation of extractive compounds into liposome particles was done by following procedure. For particle preparation 90 mg of lecithin and 10 mg of cholesterol was added to 10 mL of deionized water. In case of PHB nanoparticles 20 mg of lecithin was substituted with PHB. All of the compounds were added as chloroform solutions. Prepared mixtures were sonicated using probe ultrasound (Sonopuls, Bandelin) for 4 − 15 s. After sonication solution was placed on magnetic stirrer with heating (50 °C) till all chloroform was evaporated.

2.2.1. Particle size and colloidal stability analysis

Evaluation of particle size, polydispersity index (PdI) and colloidal stability in solution was done by standard procedure with Malvern Zetasizer Nano ZS. All samples of prepared particles were diluted 100times and 1 mL od solution was plated into the cuvette. Colloid stability was measured with same solution but with Dipp Cell (Bokrova et al., 2019). Values are represented as mean ± SD of three replicate determinations.

Table 1
Characterisation of liposome/PHB particles with different content of PHB.

Sample	d (nm)	PdI	ζ- potential (mV)
Liposomes	135.9 ± 1.27	0.207 ± 0.02	−31.3 ± 2.14
10 % PHB	252.8 ± 3.85	0.365 ± 0.03	−39.6 ± 3.53
20 % PHB	253.2 ± 1.36	0.263 ± 0.01	−36.9 ± 2.25
30 % PHB	246.2 ± 3.25	0.387 ± 0.01	−44.9 ± 0.93
50 % PHB	336.3 ± 9.96	0.257 ± 0.05	−45.1 ± 0.83
70 % PHB	245.1 ± 2.45	0.322 ± 0.03	−44.1 ± 0.78
100 % PHB	324.1 ± 11.40	0.397 ± 0.05	−25.4 ± 1.53

Note: Results are expressed as (mean ± S.D.) of three determinations.

2.2.2. Encapsulation efficiency

Encapsulation efficiency was performed as measuring of polyphenols outside the particles in solution. Samples were centrifuged for 60 min at 11 000 rpm and TPC in supernatant have been determined. Encapsulation efficiency was calculated as a percentage of encapsulated TPC to total TPC in the whole sample.

2.2.3. Imaging of nanomaterials

Prepared particles were imaged by cryogenic transmission electron microscopy (cryo-TEM) using 200 kV on FEI Tecnai F20 transmission electron microscope with 4 k CCD camera FEI Eagle, as described previously (Bokrova et al., 2019). Surface structure and diameter of PHB nanofibers was analyzed with JEOL JSM-7600 F (SEM) electron microscope.

2.3. Cytotoxicity assays

For both MTT and LDH cytototoxicity assays human epidermal keratinocyte cell line (HaCaT) was obtained from CLS Cell Lines Service GmbH (Germany). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 mg/mL of streptomycin, 100 units/mL of penicillin, and 10 % of heat-inactivated fetal bovine serum (FBS) in humidified, 5 % (v/v) CO_2 atmosphere at 37 °C.

2.3.1. MTT assay

MTT assay (Mosmann, 1983) was performed according to modified procedure of Li et al. (Li et al., 2009). Cultivation of HaCaT keratinocytes was done in DMEM (Lonza Biotec, CZ) with High Glucose, 0.4 mM L-Glutamine, without Sodium Pyruvate, with 10 % FBS and Antibiotic-Antimycotic 100x, Biosera) in cell cultivation box with atmosphere of 5 % CO_2 at 37 °C, fed every 2–3 days and passaged after reaching 80 % of confluence (Li et al., 2009). Tested particles were treated as follows. Particles were diluted 1:1 with deionized water, pipetted through 200 nm syringe filter and diluted with DMEM till the concentration range of 2–14 % (v/v).

Firstly 100 µL of properly diluted cell culture was added into 96–well plate and let in the cultivator. After 24 h medium was replaced by prepared samples in DMEM and placed back to the cultivator. As a control culture medium was used and ethanol served as a negative control. Subsequently, after another 24 h 20 µL of MTT dissolved in PBS (2.5 mg mL^{-1}) was added to each sample and incubated for 3 h in cultivator. Then 100 µL of 10 % SDS in PBS was added to each well. Plates were stored in dark and evaluated next day by ELISA Reader at 543 nm.

2.3.2. LDH assay

LDH (lactate dehydrogenase) is a stable enzyme occurring in cytosol. When cells undergo some reaction leading to the damage of plasma membrane, LDH is rapidly released to cell culture media and therefore can be determined. The LDH cytotoxicity assay was performed according to Wang et al. (2011). For this assay supernatants from exposure of cells to tested substances were used. Supernatants were used immediately or kept frozen at −80 °C. A calibration curve was prepared using sodium pyruvate/NADH solution. This solution consists of 1 mg mL·L^{-1} NADH in 0.75 mM sodium pyruvate, that were equivalent to LDH activity 0–2 000 U/mL^{-1}. For determination of LDH activity 10 µL of exposed supernatant, blank and 60 µL calibration solution was pipetted in 96–well plate in triplicates. For samples and blank 50 µL of pyruvate/NADH solution was added and incubated at

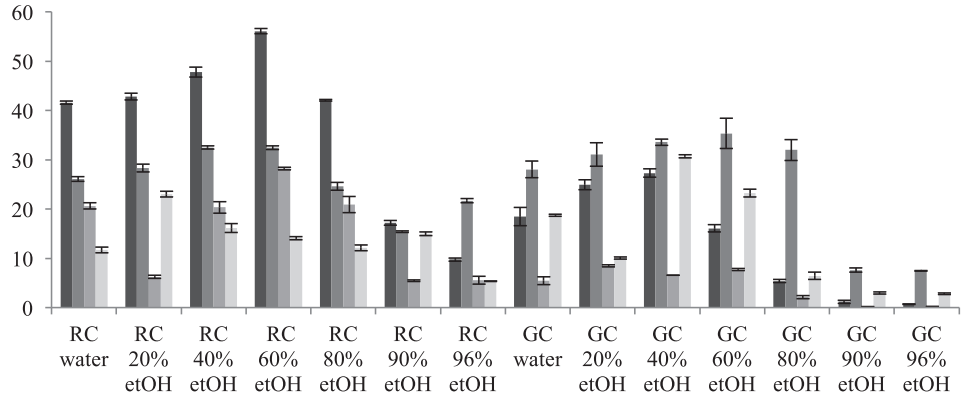

Fig. 1. Content of total phenolics and flavonoids and antioxidant activity and SPF of prepared extracts from *Coffea arabica.*
RC…roasted coffee, GC…green coffee; black columns ...total phenolics mg·g^{-1}, dark grey columns... total flavonoids mg·g^{-1}, light grey columns...antioxidant activity mg·g^{-1}, white columns...SPF.
Results are expressed as (mean ± S.D.) of three determinations.

□…Total phenolics mg.g^{-1}, □…total flavonoids mg.g^{-1}, □…antioxidant activity mg.g^{-1}, □…SPF

37 °C for 30 min. After incubation 50 µL of 4 M sodium hydroxide was added to all wells and incubated at room temperature for 5 min in dark. Last step was measuring the absorbance at 540 nm. Data are measured in absolute LDH activity units (Bokrova et al., 2019).

2.4. Statistical analysis

All experiments were performed in triplicates. Normal distribution was determined using the Shapiro-Wilk test. For hypothesis testing one-way ANOVA was used and set on significance level 0.05. Data were evaluated by Pearson correlation and expressed by correlation coefficients.

3. Results

In this work, water, ethanolic and oil extracts were prepared from *Coffea arabica*. Extracts from green coffee beans and roasted coffee beans were analyzed for their amount of phenolic compounds, total flavonoid content, antioxidant activity and Sun protection factor (SPF) according to procedures described in paragraph 2.1 and compared (Fig. 1).

3.1. Characterization of coffee extracts

All prepared coffee extracts were characterized according to phenolic compounds (TPC), flavonoid content (TEC), antioxidant capacity and value of sun protection factor. Summary results from these measurements are introduced in Fig. 1. In general, extracts from roasted coffee exhibited higher phenolics content then green coffee extracts. Regarding phenolics and flavonoid content, as the most effective extraction mixture 60 % ethanolic solution was evaluated. Nevertheless, the highest SPF was found in 20 % ethanolic solution of roasted coffee. High values of all parameters were found in pure water extracts.

Green coffee extracts in 40 % ethanolic solution exhibited the highest contents of phenolics and flavonoids. Moreover, it possessed also very good antioxidant activity and the best value of the Sun protection factor among the all tested samples. Similarly, to the roasted coffee, also in the samples of green coffee high values of all parameters were found in water extracts without ethanol. Thus, for next experiments 20 % ethanolic extract of roasted coffee and 40 % ethanolic extract of green coffee were chosen for encapsulation. Water extracts of both types of coffee were tested too due to relatively good extract compostion and advantages accompanied with using water as solvent (*e.g.* low price, no cell toxicity).

3.2. Liposome/PHB particle preparation and characterization

Combined liposome/polyhydroxy butyrate particles were prepared according to procedure described in paragraph 2.2. Prepared particles containing different amount of PHB were characterized using Zetasizer Nano ZS. In Table 1 characteristics of liposomes and PHB/liposome particles with different content of PHB are introduced and compared. The average diameter of particles was in the range of 140–340 nm. Polydisperzity index of most of combined praticles was relatively low (value 0.3), thus, particles were quite homogenous. prepared samples were measured as ζ- potential. The data in Table 1 indicate that all prepared samples had very good colloidal stability in solution. Stable particles have to possess ζ- potential below −25 mV and above +25 mV. All particles exhibited higher absolute value of ζ- potential with the only exception (100 % PHB).

3.3. Particle imaging

Prepared liposome particles were observed by cryo-TEM using procedure described in paragraph 2.2.3. We can see that with adding PHB the particle diameter increases. This observation supports the data obtained from measurement of particle diameters by ZetaSizer Nano (Table 1).

The micrographs (Image 1) show liposome particles without addition of PHB (A) and with 10–30 % PHB (B–D). These images demostrates the probable mechanism of PHB incorporation into liposome particles. The heterogenous spot structures are the PHB cores covered with the layer of phospolipid. In those particles a double function could be expected. The liposome part can serve as a transport system of encapsulated active organic UV filter, whereas the PHB component, in addition to its stabilization effect, has its own UV-scattering effect (Bokrova et al., 2019). Higher content of PHB led to increased stability; nevertheless, the manipulation with these particles is quite complicated and too rigid structure could contribute to slow release of active substances from particles to the skin. Interpretation of TEM images is quite complicated, probably PHB forms some hydrophobic core inside the particles and active substances can be encapuslated to interlayer between this core and liposome envelope. In liposome/30 % PHB particles the cores are quite large, no intermediate layer is seen and the whole structure could negatively influence encapsulation efficiency and particles rigidity. The cores are similar to the TEM image of 100 % PHB particles with no acceptable characteristics as well as carrier properties (image not shown).

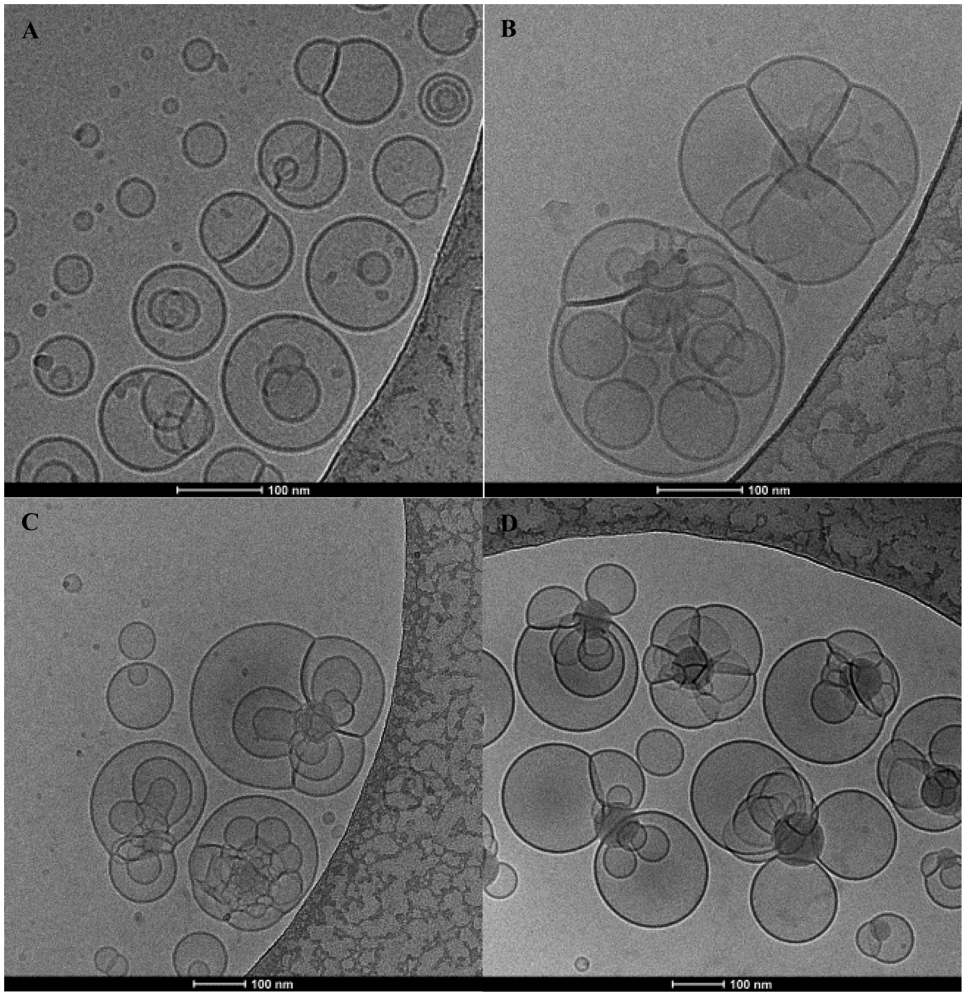

Image 1. Liposome particles imaged by cryo-TEM microscopy.
Cryo-TEM images (200 kV) of liposome particles (A) and PHB-liposome particles (B, C, D - A – liposomes, B-liposomes with 10 % of PHB, C – liposomes with 20 % of PHB, D – liposomes with 30 % of PHB). Magnification about 80 000 × .

3.4. The effect of PHB content on SPF

Effect of PHB concentration in prepared particles on Sun protection factor was studied. Presented data were measured by *in vitro* spectrophotometric procedure and calculated according to Mansur equation (paragraph 2.1.4). Results of SPF found in liposome/PHB particles with different PHB content can be seen in Fig. 2. As expected, the lowest SPF value was obtained in liposomes without addition of PHB (1.70 ± 0.06). Then the value of SPF increased with growing PHB content up to 50 %. Maximal value of SPF was measured for liposome particles with 50 % of PHB (15.13 ± 0.30) similar results were obtain for particles with 70 % of PHB. In case of 100 % PHB particles decrease

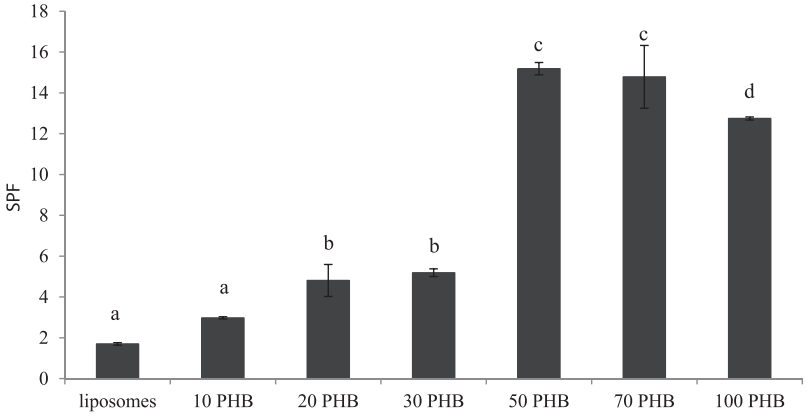

Fig. 2. Sun protection factor of empty liposome/PHB pacticles.
Results are expressed as (mean ± S.D.) of three determinations. Samples were sorted into groups by Tukey (HSD) Analysis of the differences between the categories with a confidence interval of 95 %.

Table 2
Characterization of liposome/PHB particles with encapsulated coffee extract.

Sample	d (nm)	PdI	ζ- potential (mV)	Encapsulation efficiency (EE)(% v/v)	SPF (-)
Empty liposomes	148.7 ± 0.4	0.167 ± 0.007	−41.8 ± 0.8	–	1.70 ± 0.06
Liposomes + RC water	232.6 ± 2.3	0.404 ± 0.010	−28.9 ± 1.1	41.8 ± 0.7	19.54 ± 3.92
Liposomes + RC ethanol 20 %	602.5 ± 18.5	0.470 ± 0.124	−45.7 ± 1.8	48.4 ± 1.0	20.57 ± 1.01
Liposomes + GC water	202.2 ± 7.2	0.313 ± 0.025	−32.3 ± 1.3	15.4 ± 2.1	25.60 ± 3.29
Liposomes + GC ethanol 40 %	794.6 ± 14.9	0.463 ± 0.027	−46.2 ± 0.4	60.1 ± 0.8	32.14 ± 0.64
Empty 20 % PHB liposomes	326.8 ± 9.5	0.419 ± 0.014	−41.9 ± 0.6	–	4.81 ± 1.85
Liposomes/20 % PHB + RC water	409.1 ± 9.5	0.219 ± 0.009	−47.2 ± 1.9	46.6 ± 0.1	26.60 ± 2.40
Liposomes/20 % PHB + RC ethanol 40 %	639.8 ± 1.0	0.561 ± 0.052	−45.0 ± 1.0	52.0 ± 0.9	50.45 ± 1.32
Liposomes/20 % PHB GC water	616.1 ± 16.3	0.439 ± 0.021	−43.7 ± 1.2	32.3 ± 1.3	31.09 ± 3.45
Liposomes/20 % PHB + GC ethanol 40 %	737.3 ± 0.1	0.471 ± 0.077	−47.1 ± 0.3	67.5 ± 0.2	37.65 ± 2.42
Coffee extract RC, 40 % ETOH	–	–	–	(56.8 ± 0.6)*	47.89 ± 0.62

Note: Results are expressed as (mean ± S.D.) of three determinations.
Particle size (d), polydisperzity index (PdI), colloidal stability (ζ- potential), encapsulation efficiency (entrapped phenol compounds % v/v) and sun protection factor. Values were measured immediately after mixing.
RC…roasted coffee, GC…green coffee; *…total phenolics (mg. g^{-1}).

of SPF was observed. It could have been caused due to lowered colloidal stability where particles had limit value for ζ- potential and thus tended to aggregate to higher degree.

Analyzed samples were sorted into groups by Tukey's HSD test. The same group was formed by liposomes/20 % PHB as well as liposomes/30 % PHB. Because of need of some compromise between particles UV scattering, stability and ability to release of UV absorbing substance liposomes with 20 % PHB were used for complex organic UV filters formulation.

3.5. Characterization of liposome/PHB particles with encapsulated coffee extracts

After encapsulation of coffee extracts into selected particles (liposomes with 20 % PHB) stability data vere measured by Zeta Sizer (Malvern, USA) similarly to empty particles. As introduced in Table 2, average diameter of liposome particles was in the range of 150–800 nm and combined liposome/PHB particles varied in the range of 300–740 nm. Polydispersity index was quite good (value 0.3–0.4) for all liposome samples and nearly all PHB liposomes with just several exceptions (Table 2). Samples with encapsulated coffee extracts had also very good colloidal stability in solution due to very low values of ζ-potential.

Encapsulation efficiency values in samples water and ethanolic coffee extracts packed into liposomes and PHB/liposomes were evaluated as acceptable. PHB/liposomes with 40 % ethanolic extract of green coffee exhibited the highest encapsulation efficiency (over 60 %). On the opposite, encapsulation of water extract of green coffee into both types of particles was less effective with EE 15.40 ± 2.10 for liposomes and 12.3 ± 1.3 for 20 % PHB/liposomes.

In samples of particles with encapsulated coffee extracts (Table 2) also values of sun protection factor were evaluated. The lowest SPF values were found in liposome particles with encapsulated water extract of roasted coffee (19.54 ± 3.92), while the highest SPF was determined in combined liposomes/20 % PHB with 40 % ethanolic extract of green coffee (37.6 ± 2.4). Interesting is that these particles exhibited also the highest encapsulation efficiency and very high colloid stability. This type of combination of material and natural extract seems to be the most suitable for application in some sunscreen preparative.

Particles with high values of phenolics and antioxidant activity were tested for short-term (1 h) and long-term (2 months) stability of SPF value and compared with crude coffee extracts (Table 3). In general, liposomes with PHB were more stable and exhibited higher SPF value after 1 h and 2 months in darkness at 8 °C. While SPF of ethanolic roasted coffee extract (47.89 ± 0.6) dropped during 1 h storage to about one half of original SPF value (26.15 ± 0.8), in encapsulated form the value of SPF was substantially more stable. After 10 % decrese during 1 h (45.10 ± 1.3) the SPF value dropped during 2 months of storage to about 72 % only (Table 3). Liposomes without PHB lost during 2month storage about 30–70 % of SPF and some of them also partially their size, stability and encapsulation efficiency (Table 3). Addition of 20 % PHB resulted in quite unchanged value of colloid stability (ζ- potential), small changes in size and PdI, small decrease of encapsulation efficiency and no more than 30 % decrease of SPF. The highest SPF value was found in liposome/20 % PHB particles with 40 % ethanolic extract from roasted coffee (37.6 ± 0.3).

3.6. Cytotoxicity testing with MTT assay

Particles with different amount of PHB as well as particles with coffee extracts were further examined for cytotoxicity to evaluate safety of these structures to skin cells. Cytotoxic efffect was tested on human keratinocytes (HaCaT) cell line according to 2.3.1. Results of MTT assay of liposome/PHB particles with PHB content in the range of 0–100 % are displayed in Table 4. Results of cytotoxicity of empty particles were compared with cytotoxicity of particles with different coffee extracts. Values of empty liposomes and liposomes with coffee extracte were compared as displayed in Fig. 3, while Fig. 4 summarizes cytotoxicity of empty and filled liposomes/20 % PHB.

The highest cell viability compared to control exhibited sample with 2% (v/v) concentration of liposome/20 % PHB particles in culture medium. Oppositely, the most negative influence on cell viability was found in the sample containing 14 % (v/v) of liposomes/70 % PHB. Liposomes/20 % PHB exhibited no significant or slightly positive influence on the cell viability, which was not affected by further increasing of particle concentration. Liposomes/70 % PHB and liposomes/100 % PHB negatively affected cell viability. This effect was more intensive with increasing particle concentration as demonstrated by low p value ($p \leq 0.000\ 1$; Table 4).

For evaluation of correlation of increasing particle concentration with cell viability Pearson correlation was calculated (Table 5). Moderate negative correlation between concentration of particles and cell viability was observed in all types of particles with exception of liposome/20 % PHB and liposomes/20 % PHB with encapsulated water green coffee extract. Moderate positive correlation was observed in samples with green coffee extracts from into liposomes only. Positive effect of these particles on cell viability could be explained as influence of phenolics released from gradually degraded liposomes.

Cell viability in presence of liposome particles with either water or ethanolic extract of green coffee has not significantly differed when compared to control (Table 3, Fig. 4). Liposome particles with coffee extract exhibited generally higher values of cell viability in cell culture. Addition of PHB led to lower cell viability (Fig. 4). Nevertheless, PHB liposome particles showed a significant reduction in mitochondrial

Table 3
Stability of SPF of free and encapsulated coffee extract after 2-month storage.

Sample	d (nm)	PdI	ζ- potential (mV)	Encapsulation efficiency (EE)(% v/v)	SPF (-) After 2 months
Empty liposomes	228.4 ± 1.3	0.370 ± 0.007	−36.0 ± 1.7	–	1.35 ± 0.11
Liposomes + RC water	454.1 ± 32.2	0.867 ± 0.074	−20.9 ± 0.3	43.5 ± 0.8	10.12 ± 3.92
Liposomes + RC ethanol 20 %	744.5 ± 17.1	0.464 ± 0.102	−31.4 ± 1.7	51.5 ± 1.1	13.87 ± 0.95
Liposomes + GC water	198.2 ± 1.7	0.175 ± 0.005	−27.8 ± 0.5	13.0 ± 0.1	6.60 ± 3.29
Liposomes + GC ethanol 40 %	704.5 ± 2.5	0.423 ± 0.032	−49.4 ± 1.2	46.6 ± 0.3	18.14 ± 0.64
Empty 20 % PHB liposomes	182.9 ± 3.3	0.397 ± 0.034	−40.8 ± 1.5	–	5.5 ± 0.3
Liposomes/20 % PHB RC water	387.5 ± 2.2	0.307 ± 0.036	−36.9 ± 0.6	54.7 ± 1.7	21.6 ± 0.8
Liposomes/20 % PHB RC ethanol 40 %	731.0 ± 23.8	0.401 ± 0.162	−40.5 ± 0.2	62.9 ± 2.1	37.6 ± 0.3
Liposomes/20 % PHB GC water	319.3 ± 13.3	0.120 ± 0.038	−48.3 ± 0.9	36.5 ± 1.0	22.1 ± 2.1
Liposomes/20 % PHB + GC ethanol 40 %	658.3 ± 0.2	0.412 ± 0.054	−44.5 ± 0.8	65.2 ± 0.6	27.5 ± 2.4
Coffee extract RC, 40 % ETOH	–	–	–	(4.3 ± 0.6)*	1.9 0.1

Note: Particle size (d), polydisperzity index (PdI), colloidal stability (ζ- potential), encapsulation efficiency (entrapped phenol compounds % v/v) and sun protection factor. Values were measured immediately after mixing. Results are expressed as (mean ± S.D.) of three determinations.
RC…roasted coffee, GC…green coffee; *…total phenolics (mg. g^{-1}).

succinate dehydrogenase activity in HaCaT cells after exposure with 10 % (0.20 vol concentration) ($p < 0.05$), 12 % (0.24) and 14 % (0.28) volume concentrations ($p < 0.01$). Significant difference was found between liposome and PHB liposome particles on HaCaT cell metabolic activity after 24 h at all concentrations used.

In liposome/20 % PHB particles with both water and ethanolic green coffee extracts at least two concentrations of added particles were found significantly different from the control (Fig. 4). Addition of green coffee extract slightly decreased negative influence of particles to human cells. Liposome/20 % PHB with green coffee can be considered as safe up to 8% concentration of particles in medium (0.16 vol concentration).

3.7. Cytotoxicity testing with LDH assay

As another tool for evaluation of influence of the sample to the cell survival, LDH assay was performed according to 2.3. Data are showed in Table 6. Lowest LDH activity, and, thus, low effect on cell viability exhibited sample with 10 % of liposomes/10 % PHB (77.0 ± 40.3). Sample with liposomes/30 % PHB showed the highest LDH activity at higher concentration. The results of LDH Assay suggest slight effects of liposome particles on LDH release from HaCaT cells when compared to control. No significant difference ($p < 0.05$) of LDH release was demonstrated in liposomes and liposomes/10 % PHB particles at all concentrations used. A significant difference ($p < 0.05$) between effect of liposomes and liposomes/PHB particles was found at whole concentration range in presence of 20 % of PHB and more.

Samples evaluated in Table 6 were sorted into groups by Tukey (HSD) Analysis of the differences between the categories with a confidence interval of 95 % and marked by letters (a, b, c, d and e). Effect of PHB amount on cell viability was analysed by analysis of variance.

For determination whether increasing concentration of particles has correlated with LDH activity Pearson correlation was calculated (Table 5). Moderate positive correlation between increasing sample concentration and LDH activity was observed in samples of liposomes and liposomes/10 % PHB and liposomes/50 % of PHB. Other samples showed high positive correlation between increasing sample concentration and LDH activity (Table 7).

4. Discussion

The aim of present study was to fabricate and charecterise newly proposed combined PHB/liposomes with natural phenolic compounds as organic UV filters. This work is based on two recent studies. The first one was focused on role of PHA granules in bacterial cells. This study has demonstrated that intracellular PHA granules naturally formed in bacterial cells efficiently scatter UV radiation and serve in PHB producing bacteria as protective mechanism against harmful effect of UV radiation and reactive oxygen species (Slaninova et al., 2018). In second study, novel PHB-liposome nanoparticles were synthesised and their toxicity and/or biocompatability was characterized (Bokrova et al., 2019). This study demostrates that addition of PHB during liposome preparation led to incorporation of polymer into particles preserving their size, polydispersity index and stability (Soema et al., 2015). Cytotoxicity studies *in vitro* showed that effect of exposure of human keratinocytes to PHB-liposomes significantly differed when compared to liposomes. Interestingly, novel combined PHB liposomes were found less cytotoxic for HEK and HaCaT cells. Surprisingly, also pro-inflammatory effects of liposomes as tested by production of interleukin-8 (Brown et al., 2001) was significantly lower in presence of PHB in liposomes structure. Thus, combined PHB-particles were proposed as active carriers for many organic compounds such as antioxidants, anti-ageing substances and other bioactive components present in natural extracts (Bokrova et al., 2019).

It can be suggested that the combined liposome-PHB particles could take advantage especially as carriers in topical applications with

Table 4
MTT results for particles with different content of poly-3-hydroxybutyrate (PHB).

Cell viability (%)	Particle content (% v/v)								
Samples	0	2	4	6	8	10	12	14	P value
liposomes	77.0^{a} ± 18.3	80.0^{ab} ± 3.5	65.0^{ab} ± 5.6	60.4^{bc} ± 4.2	62.4^{bc} ± 4.7	53.6^{bc} ± 2.1	46.9^{bc} ± 2.6	52.4^{c} ± 7.4	***
10 % PHB	77.0^{a} ± 18.3	73.9^{a} ± 5.4	65.1^{b} ± 1.3	58.0^{bc} ± 2.4	53.5^{cd} ± 1.4	49.4^{d} ± 0.7	46.2^{de} ± 0.8	39.3^{e} ± 0.8	***
20 % PHB	77.0^{bc} ± 18.3	97.2^{a} ± 2.0	90.3^{ab} ± 4.0	77.2^{bc} ± 5.3	76.7^{bc} ± 4.1	82.0^{ab} ± 7.6	79.1^{ab} ± 6.4	70.1^{d} ± 5.2	n.s.
30 % PHB	77.0^{a} ± 18.3	85.7^{a} ± 18.9	80.5^{ab} ± 10.5	65.1^{ab} ± 8.4	58.8^{ab} ± 4.1	58.9^{ab} ± 6.7	57.7^{ab} ± 3.4	45.1^{b} ± 6.3	**
50 % PHB	77.0^{bc} ± 18.3	70.8^{b} ± 6.9	60.5^{bc} ± 0.5	57.0^{bc} ± 1.5	57.6^{bc} ± 0.9	54.1^{c} ± 1.9	89.7^{a} ± 9.4	48.2^{c} ± 2.0	*
70 % PHB	77.0^{a} ± 18.3	64.3^{a} ± 4.8	57.7^{ab} ± 5.6	52.3^{abc} ± 3.8	45.6^{bc} ± 2.8	44.8^{bc} ± 3.7	40.1^{c} ± 2.9	37.6^{c} ± 2.7	***
100 % PHB	77.0^{a} ± 18.3	65.4^{ab} ± 6.8	58.2^{b} ± 2.8	61.1^{b} ± 4.3	44.6^{c} ± 1.6	41.4^{c} ± 1.9	39.8^{c} ± 0.8	38.6^{c} ± 2.3	***

Note: Results are expressed as (mean ± S.D.) of three determinations.
Samples were sorted into groups by Tukey (HSD) Analysis of the differences between the categories with a confidence interval of 95 % marked by letters (a, b, c, d and e). Samples on different significant levels are marked as follows * $p \leq 0.05$, ** $p \leq 0.001$ and *** $p \leq 0.000\ 1$ and n.s. for not significant results.

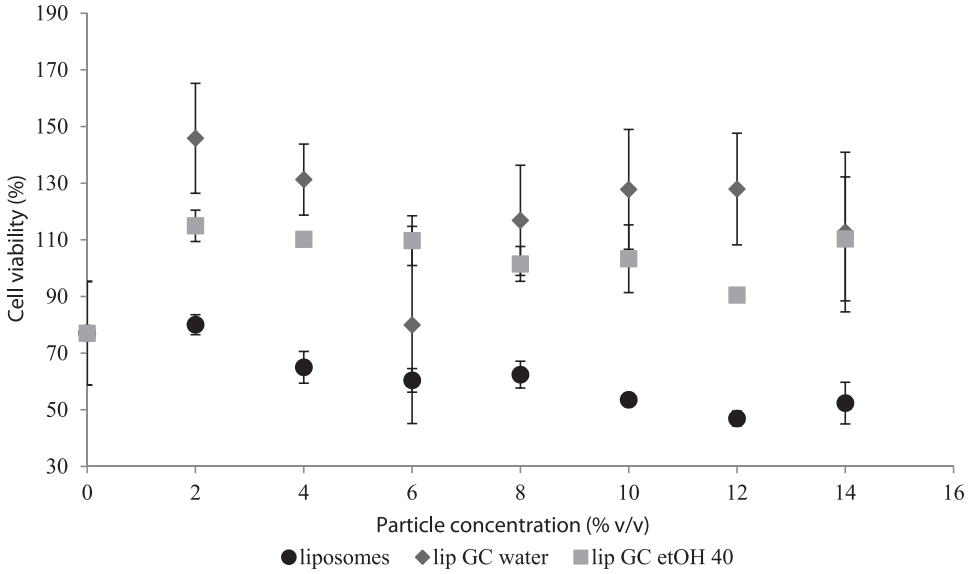

Fig. 3. Cell viability results from MTT assay for liposomes and liposomes with water and ethanolic (40 %) extracts from green coffee.
Note: lip GC Water…liposomes with encapsulated water extract of green coffee; lip GC etOH 40…liposomes with encapsulated ethanolic (40 %) extract of green coffee
Results are expressed as (mean ± S.D.) of three determinations. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

additional UV-scattering effect caused by PHB. Therefore, in this study PHB/liposome particles will be used to development of a new UV protective system by using encapsulation of extracts from *Coffea arabica* used as a model organic sunscreens. Extracts from *Coffea arabica* contain high amount of active substances of phenolic type with potential UV absorption and antioxidant properties (Hečimovič et al., 2011).

The successful use of PHB-liposomes with encapsulated coffee extract thorough detailed characterization and quantitation of active substances and sun protection properties, but also testing of their interactions with human cells. Screening the *in vitro* toxicity of newly fabricated UV scattering nanoparticles with encapsulated phenolics can be considered as an efficient strategy. This study therefore aimed to test the benefitial as well as toxic effects of novel PHB-liposome based organic sunscreens.

Choice of coffee as a model of natural sunscreen was determined by very high content of active phenolic compounds and high SPF value. Of course, liposome/PHB particles could be prepared with any compound or complex natural extract with similar properties and SPF. First, extracts of green and roasted coffee were prepared and characterized. Differences in values of total phenolics, antioxidant activity and SPF were found between roasted and green coffee; in roasted coffee mostly higher values of above mentioned parameters were determined. The reason is that the quality and composition of coffee extract is strongly related to the composition of the roasted beans, which is affected by composition of green beans as well as by post-harvesting processes such as drying, roasting and grinding. During roasting, natural phenolic antioxidants present in coffee (such as chlorogenic acid) undergo intensive degradation, which results in a decrease of total antioxidant capacity. However, during roasting proces another types of antioxidant compounds, such as Maillard reaction products are formed, which can again enhance the antioxidant activity of coffee extract (Hečimovič et al., 2011), as documented also in present study.

Previously was demonstrated that colloid carriers are capable to enhance the accumulation of the sunscreen at the skin. Further, they can contribute to increased water resistance, sun protection factor (SPF) and photostability of UV filters (Müller et al., 2002a). Thus, newly prepared particles with different PHB content and encapsulated coffee extracts were characterized in terms of their size and colloid stability. Appearance of the particles was observed using cryo-TEM imaging, which demostrated the incorporation of PHB into liposome particles. The size of PHB-liposomes fluctuates around 200 − 300 nm and colloid stability was over −45 mV. Based on stability data and values of SPF during long-term storage it can be claimed that newly constructed PHB-liposome particles can be considered as promising system for multifunctional and safe topical application. Stable structure of proposed formulation, excellent biocompatibility and biodegradability allowed i) time limited release of organic UV filters at the skin surface and ii) subsequent ful biodegradation of combined particles. Moreover, additional UV-protective effect of PHB could enlarge the application potential of combined particles preferentially in topical sunscreens

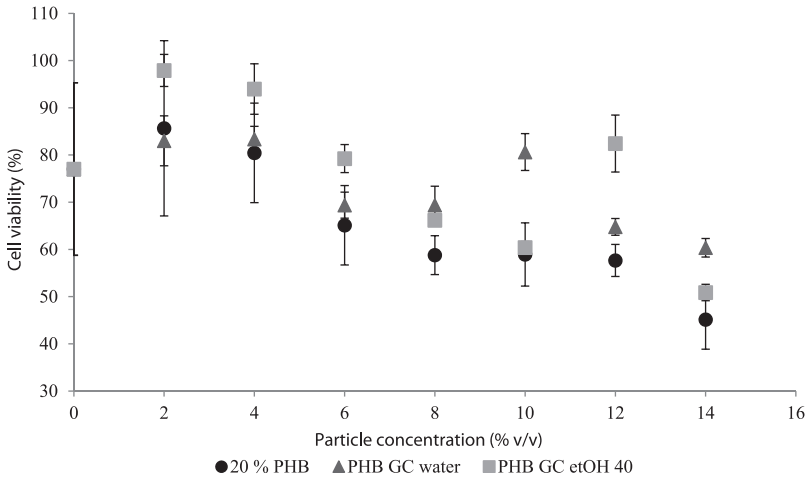

Fig. 4. Cell viability results from MTT assay for liposomes and liposomes/20 % PHB with ethanolic extracts of green coffee.
Note: 20 % PHB…liposomes/20 % PHB; PHB GC Water…liposomes/20 % PHB with encapsulated water extract of green coffee; PHB GC etOH 40…liposomes/20 % PHB with encapsulated ethanolic (40 %) extract of green coffee
Results are expressed as (mean ± S.D.) of three determinations. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

Table 5
: Pearson correlation between concentration of particles and cell viability determined by MTT assay.

Pearson correlation	liposomes	10 % PHB	20 % PHB	30 % PHB	50 % PHB	70 % PHB	100 % PHB	lip GC water	lip GC EtOH 40 %	PHB GC water	PHB GC EtOH 40 %
MTT assay	**−0.5579**	**−0.6588**	−0,025	**−0.5010**	**−0.3458**	**−0.6996**	**−0.6987**	**0.5177**	**0.4808**	−0.2518	**−0.2761**

Note: Values in bold are different from 0 with a significance level α = 0.05.
GC…green coffee; lip GC water… liposomes with encapsulated water extract of green coffee; lip GC etOH 40…liposomes with encapsulated ethanolic (40 %) extract of green coffee; PHB GC Water…liposomes/20 % PHB with encapsulated water extract of green coffee; PHB GC etOH 40…liposomes/20 % PHB with encapsulated ethanolic (40 %) extract of green coffee.

Table 6
LDH activity of particles.

LDH activity (U/mL)	Particle content (% v/v)								
Samples	0	2	4	6	8	10	12	14	P vavalueue
liposomes	80.0^{a} ± 66.7	140.0^{a} ± 27.7	136.2^{a} ± 18.9	198.6^{a} ± 18.3	162.6^{a} ± 67.0	138.9^{a} ± 12.7	138.3^{a} ± 100.2	161.5^{a} ± 42.3	*
10 % PHB	80.0^{b} ± 66.7	162.6^{ab} ± 60.3	188.9^{ab} ± 10.8	173.3^{ab} ± 19.1	103.4^{ab} ± 31.7	77.0^{b} ± 40.3	243.3^{a} ± 30.4	165.3^{ab} ± 116.4	*
20 % PHB	80.0^{b} ± 66.7	158.3^{ab} ± 54.8	180.3^{ab} ± 79.0	197.6^{ab} ± 97.1	313.8^{a} ± 22.7	262.7^{a} ± 82.1	261.6^{a} ± 118.1	246.0^{ab} ± 72.8	***
30 %PHB	80.0^{e} ± 66.7	183.0^{e} ± 49.4	241.2^{de} ± 24.5	335.5^{cd} ± 90.5	369.4^{c} ± 56.4	467.9^{bc} ± 120.1	440.7^{a} ± 96.4	409.6^{a} ± 598	***
50 % PHB	80.0^{a} ± 66.7	135.7^{a} ± 65.9	133.5^{a} ± 77.2	189.5^{a} ± 40.9	210.5^{a} ± 22.5	168.0^{a} ± 68.8	139.4^{a} ± 46.3	206.1^{a} ± 55.2	**
70 % PHB	80.0^{b} ± 66.7	297.1^{a} ± 26.9	216.4^{a} ± 43.6	212.6^{a} ± 19.8	201.3^{ab} ± 53.4	199.2^{ab} ± 19.2	276.7^{a} ± 64.0	253.0^{a} ± 11.4	***
100 % PHB	80.0^{b} ± 66.7	211.6^{a} ± 32.2	168.5^{ab} ± 37.6	204.0^{a} ± 37.6	223.9^{a} ± 9.7	260.0^{a} ± 27.7	225.5^{a} ± 23.2	286.9^{a} ± 40.9	***

Note: Results are expressed as (mean ± S.D.) of three determinations.
Samples were sorted into groups by Tukey (HSD) Analysis of the differences between the categories with a confidence interval of 95 % marked by letters (a, b, c, d and e). Samples on different significant levels are marked as follows * p ≤ 0.05, ** p ≤ 0.001 and *** p ≤ 0.000 1 and n.s. for not significant results.

Table 7
Pearson correlation between particle type and cytotoxicity assays.

Pearson corelation	liposomes	10 % PHB	20 % PHB	30 % PHB	50 % PHB	70 % PHB	100 % PHB
LDH assay	**0.4216**	**0.3978**	**0.6773**	**0.8594**	**0.5306**	**0.6318**	**0.7661**

Note: Values in bold are different from 0 with a significance level α = 0.05.

(Slaninova et al., 2019). Above mentioned reduced inflammatory effect of liposomes with addition of PHB (Bokrova et al., 2019) is very important in sunscreens because of adverse processes contributing to inflammation manifested after UV exposure (Elsabahy and Wooley, 2012).

Because of testing safety of functionalized liposome/PHB particles, immortalized HaCaT keratinocytes were used for this study (Goreham et al., 2018). It can be stated that the cytotoxity of the proposed functionalized particles at concentrations tested was low. Cell viability in presence of liposomes with either water or ethanolic extract of green coffee was generally higher than in presence of empty liposomes. Liposome/PHB particles showed a significant reduction in mitochondrial SDH activity in MTT test ralized with HaCaT cells after exposure to 10 % and higher particles concentrations. Different effect was found between liposomes and PHB-liposomes on metabolic activity of HaCaT cells after 24 h exposure to concentrations higher than 10 %, which is the concentration usually added to cosmetic formulas. In liposome/20 % PHB particles addition of green coffee extract slightly decreased negative influence of particles to human cells. Liposome/20 % PHB with green coffee can be considered as safe up to 10 % concentration of particles in medium (0.20 vol concentration).

As another tool for evaluation of influence of the sample to the cell survival, LDH assay was performed. According to the results of extracellular lactate dehydrogenase activity slight effects of empty liposomes on LDH release from keratinocytes was evaluated when compared to control. A significant difference ($p < 0.05$) between effect of liposomes and PHB-liposomes was found at whole concentration range in presence of 20 % of PHB and more. Nevertheless, according to LDH assay both particles can be considered as safe.

Due to the results of present study we can conclude, that newly fabricated combined PHB-liposomes can be safely used as functional and stable carriers of organic UV filters for topical sunscreens with additional UV-scattering effect of PHB. Further study is needed for deeper understanding of interactions between combined PHB-liposomes and their UV-protecting components and human skin *in vivo*.

5. Conclusions

In conlusion, the results of the present study confirm that new UV protecting systems based on liposomes/PHB were fabricated and characterized. PHB content was tested in the range of 0–100 %. As model organic UV filters extracts of green and roasted coffee with high phenolics content, high antioxidant activity and sun protection factor value about 40–50 were encapsulated into liposomes and liposomes/PHB. Based on experimental data, novel PHB-liposome particles can be considered as promising sunscreen system for topical application. Incorporation of 20 % PHB into liposome preserved the optimum size and stability of particles, reduced inflammation and enhanced UV-protective effect. Addition of PHB into liposomes led to increased stability during long-term storage. Cytotoxicity studies showed significant differences in cell viability after exposure to pure liposomes and PHB-liposomes *in vitro*. Combined PHB-liposome particles of the new type were not found as toxic for HaCaT cells and in LDH test up to 12 % concentration. Newly fabricated particles could be used as active carriers for encapsulation of active sunscreen components of natural origin such as organic UV filters, antioxidants, antiinflammatory substances and many others, combined with additional UV-scattering effect of PHB.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

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