Nanoencapsulation of propolis polyphenols and its biochemical activity for skin applications

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Introduction:

Interest in natural products, especially bee products, has increased in recent years to promote health and well-being for humans. Bee products such as honey, propolis, royal jelly, bee pollen, wax and venom have been extensively researched for their healing properties [1, 2, 3, 4]. Propolis, a resinous substance, presents many biological and pharmacological properties, such as immunostimulatory, antibacterial, antiseptic, anti-inflammatory, and antioxidant. It has been used for centuries in traditional medicine and in recent years in the manufacturing of functional foods and in the cosmetics industry [2, 5, 6]. Propolis contains resins, waxes, aromatic components, pollen, and other components, with polyphenols and terpenes to be mainly bioactive [2, 7, 8, 9, 10, 11, 12, 13]. Because of the low solubility of active ingredients of propolis in natural solvents, the extraction and formulation process are a challenge [14, 15]. Different extracts have been developed to increase extraction efficiency [16, 17]. For the use of propolis in medicinal products, dietary supplements and foods, the ingredients have been encapsulated in various carriers [18, 19, 20]. Various carriers have been used to trap propolis, such as zein/caseinate/alginate nanoparticles, chitosan etc. [20]. Liposomes and cyclodextrins have been also used in order to encapsulate propolis ingredients [21, 22, 23, 24] and showed an increase in activity (antioxidant, antimicrobial) as well as stability [25, 26, 27]. In the present study, simultaneous extraction and entrapment were performed in a cyclodextrinliposome system, with aim of the *in vitro* study of the controlled release of polyphenols and antioxidant capacity of the propolis extract.

Materials & Methods:

Propolis extraction preparation
To produce the extract, 10% (w/w) raw propolis (organic certified from Greek cultivation), as solvents 1.3 propanediol (Connect Chemicals, Italy), ultrapure water at ratio 45/55 and 5% (w/w) hydroxypropyl-β-cyclodextrin (Gangwal Chemicals Pvt. Ltd., India) were used. The propolis was removed, after seven days and the extract was filtered by 25μm nylon bag. The 3% w/w liposome, Pro-LipoTM Neo (Lucas Meyer Cosmetics, France), was added under intense stirring (3000rpm). After three days (at 6oC), the extract was filtered through a 0.22μm Millipore membrane filter and stored at 6 °C.

Total Phenolic Content (TPC)
The total phenolic content was determined using the Folin–Ciocalteu method according to the method by Arnous et al. 2002 [28].

Antioxidant Activity by 2,2-diphenylpicrylhydrazyl (DPPH)
The antioxidant activity of the propolis extract, was determined by using 2,2-diphenylpicrylhydrazyl (DPPH) free radical as it is described by Brand-Williams et al. 1995 [29].

Stability of propolis extracts
Propolis extract was stored for six months at different temperatures (room temperature, 6°C and 38°C) for physical and chemical stability assessment. Refractive index (% Brix) (RX-5000α, ATAGO CO., Japan), pH (Seven Compact, Mettler Toledo), density (DMA 38, Austria) and organoleptic characteristics like aspect, color and odor were determined for the physical stability. The parameters that were tested for chemical stability were total phenolic compounds by the Folin–Ciocalteu method
(Merck KGaA, Darmstadt, Germany) and antioxidant activity by DPPH (Sigma-Aldrich, MI, USA) scavenging assay.

In vitro controlled release study
In vitro release study of the polyphenols of propolis was measured in a buffer solution pH 7.2 (CARLO ERBA Reagents, Val-de-Reuil, France) at 37 °C, by using dialysis sacks MWCO = 1000 (Pur-A-Lyzer, Midi Dialysis Kit, Sigma-Aldrich). Polyphenol concentration and antioxidant activity were examined at different time points (from 15min to 48h).

Measurement of Intracellular ATP Levels
We assessed the effect of propolis extract on the viability of fibroblasts based on intracellular ATP determination. ViaLight HS BioAssay kit (Lonza, Walkersville, MD, USA) was used for measuring ATP, according to the manufacturer's protocol. Briefly, 3.5 × 103 NHDF cells per well were seeded in 96-well microplates 24 h prior to the experiment and then treated with different CRPP concentrations (0%–3% v/v), prepared in culture medium. After a 48h incubation, ATP intracellular levels were determined in a GloMax 20/20 single-tube luminometer (Promega) for 1s.

RNA Isolation and cDNA Synthesis- NHDF Cells
Total RNA was isolated from primary normal human dermal fibroblasts using a kit by Norgen BIOTEK Corporation (ON, Canada) according to manufacturer's instructions. Then it was quantified using a NanoDrop at 260/230 nm and 260/280 nm. The RNA quality was confirmed on 1% agarose gel. cDNA was synthesized from 0.8 μg total RNA using reversed transcription according to Super-Script II (Invitrogen, Paisley, United Kingdom) protocol. The reaction mixture was incubated at 42 °C for 50 min, followed by heat inactivation at 70 °C for 15 min using oligo (dT) primers.

Primer Design and RT-PCR Analysis
Target cDNAs were amplified using gene specific primers and designed from the transcribed region of each gene using Primer Express 1.5 software (Applied Biosystems, Darmstadt, Germany). Quantitative RT-PCR reactions were performed on the Stratagene MX3005P using iTaq™ fast SYBR® Green supermix with ROX (BioRad Laboratories, Hercules, CA, USA), gene specific primers at a final concentration of 0.5 μM each and 1 μl of the cDNA as template. Detailed information for all the genes in this study, including names, , accession numbers, gene symbols, gene specific primers and predicted topology, are presented in supplemental data set. PCR cycling started at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The primer specificity and the formation of primers-dimers were monitored by dissociation curve analysis and agarose gel electrophoresis on a 4% (w/v) gel. As genes internal standards was used glyceraldehyde-3-phosphate dehydrogenase (GADPH) and Actin beta (ACTB). Relative transcript levels of the gene of interest (X) were calculated as a ratio to the internal standard gene transcripts (H), as (1 + E) – ΔCt , where ΔCt was calculated as (CtX – CtH). For each amplicon PCR efficiency (E) was calculated employing the linear regression method on the log (fluorescence) per cycle number data, using LinRegPCR software. Three biological repeats were performed for all real-time quantitative PCRs (qPCRs).

Propolis extracts Incorporated in an Emulsion—Reconstructed Human Skin Model
We evaluated the potential skin irritation of cosmetic formulation based on propolis extract with reconstructed human skin model. The cosmetic formulation was an oil in water basic cream base with 1% propolis extract incorporated. The parameters measured *in vitro* were the percentage cell viability using an MTT effective time 50 (ET50) assay after topical application of the products for different exposure times (2 h, 5 h and 18 h) according to manufacturer's instructions. For negative control Dulbecco's phosphate-buffered saline (DPBS) was used as well as for positive control 5% sodium dodecyl sulfate (SDS) was used. For negative control, 1.0% Triton X-100 was used.

Statistical Analysis
Results are expressed as the mean ± SD of three different experiments. Statistical analysis between two individual groups was performed by Student's t-test. A $p \leq 0.05$ was considered statistically
significant. Statistical analysis and graphs were performed with GraphPad Prism 5 and Sigma Plot Software v.10.

Results & Discussion:

Characterization of propolis extract
The physicochemical stability test revealed a possible degradation of phenolic compounds after the end of the first four weeks, showing a statistically significant deviation from the initial value. The temperature of 6 ºC, seems to be the most stable, as the there was no statistically significant difference between week 4 and 8 (Table 1). The propolis extract was stable in its physical parameters for 24 weeks, as the values were not changing by more than 15% compared to the initial value.

Table 1. Chemical stability test of DPPH: Antioxidant Activity (±SD) and TPC: Total Phenolic Compounds (±SD), of propolis extract. Values with the same letter in the same column do not show a statistically significant difference, $p \leq 0.001$.

Week	Parameters	DPPH (mM Trolox equiv.)	TPC (μg GA/ml)
T(0)		33.67 ± 0.16	100.9 ± 0.7
4th week	RT	29.11^{d,e,f} ± 0.34	88.2^{b,d,e} ± 1.7
	6ºC	29.38^{a,b,c,d} ± 0.49	88.9^{c,d,e} ± 1.7
	38ºC	30.22^{a} ± 0.23	80.7^{h} ± 1.2
8th week	RT	27.69^{h} ± 0.58	85.2^{a,d,f,g} ± 1.2
	6ºC	28.28^{b,e,g,h} ± 0.67	88.9^{a,b,c} ± 0.34
	38ºC	28.84^{c,f,g} ± 0.79	79.4^{h} ± 2.2
24th week	RT	22.13 ± 0.72	70.9 ± 0.7
	6ºC	23.90 ± 0.23	84.5^{g} ± 3.9
	38ºC	25.62 ± 0.47	61.6 ± 0.35

In vitro controlled release studies
In the *in vitro* release study of propolis extract, the antioxidant activity (DPPH) and the total phenolic compounds (TPC) reach a plateau at 8 hours (25-60%) (Figure 1).

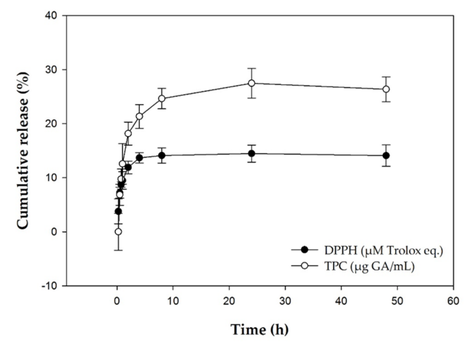

Figure 1. *In vitro* release of total phenolic compounds (TPC) and antioxidant activity (DPPH) of Propolis extract for time 0 to 48h. The results are shown as the mean ±SD of three experiments.

Moreover, we assessed the effect of propolis extract (CRPP) on the viability of NHDF cells based on intracellular ATP determination. NHDF cells were incubated for 48 h with three different concentrations of CRPP (0.01%, 0.1% and 1%) have shown an increase to the intracellular levels of ATP up to 100% compared to untreated cells (control) (Figure 2).

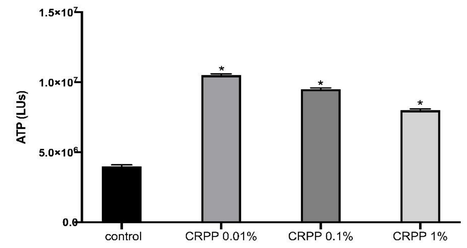

Figure 2. Intracellular levels of ATP (LUs). NDHF cells were incubated for 48 h with three different concentrations (0.01%, 0.1% and 1%) of CRPP. The results are shown as the mean ± SD of three independent experiments. *—$p \leq 0.05$, significantly different from untreated cells.

To understand the molecular mechanisms associated with CRPP bioactivity, an RT-qPCR platform for an array of key-genes was deployed. Genes involved (number of total genes = 20) in primary cellular processes in human skin, such as cell proliferation, anti-oxidant and immune response, aging, inflammation and extracellular matrix (ECM) generation were assessed. Here we present, specific gene transcript levels showed significant changes under the experimental conditions (Figure 3). According to Figure 3, transcript levels of genes encoding proteins, tumor necrosis factor (TNFα) human aquaporin-3 (AQP3), interleukin-4 (Il-4), vascular endothelial growth factor A (VEGFα) and integrin subunit beta 2 (ITGB2) were upregulated with the addition of CRPP in NHDF cells compared to untreated NHDF cells (control) ($p < 0.05$).

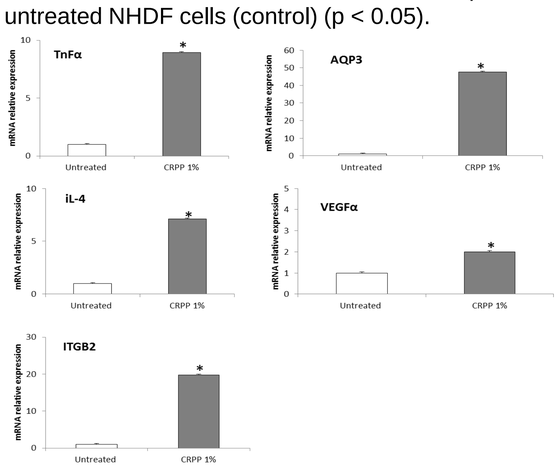

Figure 3. Expression analysis of candidate genes in the presence and absence of 1% v/v CRPP in NHDF cells. Transcript levels of genes encoding proteins, tumor necrosis factor (TNFα) human aquaporin-3 (AQP3), interleukin-4 (Il-4), vascular endothelial growth factor A (VEGFα) and integrin subunit beta 2 (ITGB2) are showed. Data corresponds to the mean ± SEM of at least three independent experiments. Gene expression levels were normalized in relation to intact cells and using the geometric mean of two reference genes (βactin and glyceraldehyde-3-phosphate dehydrogenase (GADPH)). * denote significantly different from untreated NHDF cells (control) of $p < 0.05$ (two tailed unpaired student t-test), respectively. Lastly, we evaluated the potential skin irritation of a cosmetic formulation based on CRPP. The cosmetic formulation was an oil in water basic cream base with 1% CRPP incorporated (Figure 10). The parameters measured in vitro were the percentage cell viability using an MTT ET50 assay after topical application of the products for different exposure times (2 h, 5 h and 18 h) according to manufacturer's instructions. For negative control DPBS was used as well as for positive control 5% SDS was used. For negative control, 1.0% Triton X-100 was used.

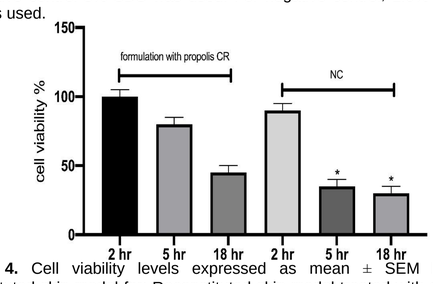

Figure 4. Cell viability levels expressed as mean ± SEM based on reconstituted skin model for: Reconstituted skin model treated with a cosmetic formulation with 1% CRPP and 1% triton-100 as negative control (NC). *—$p < 0.05$ significantly different from the formulation.

A system carrying propolis in cyclodextrins and liposomes for skin applications was developed. The propolis extract was physicochemically stable for 24 weeks at 6ºC. Furthermore the system was stable as well as in total phenolic compounds and antioxidant activity. The controlled release study revealed that at 8 hours, the cumulative release was 82%. The anti-aging, antioxidant and antibacterial activities of propolis polyphenols were retained to levels similar and comparable to those of propolis methanolic extracts, making the system ideal for applications where non-toxic solvents are required and controlled release of the polyphenol content is desired.

Conclusions:

In the present study, the polyphenols of propolis were loaded in a system consisting of cyclodextrins and liposomes, mainly for skin applications. The propolis extract was physicochemical stable and the ingredients of propolis - polyphenols- were protected. The polyphenols can be released at a controlled rate when placed in a suitable environment. Our results demonstrate that the encapsulation and delivery system can retain the original anti-mutagenic, anti-oxidative and anti-ageing effects of propolis polyphenols to levels similar and comparable to those of propolis methanolic extracts of similar geographic origin, making the system ideal for applications where non-toxic solvents are required, and controlled release of the polyphenol content is desired.

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