THE USE OF BIOACTIVE COMPOUNDS AND THEIR ENCAPSULATION INTO LIPOSOMES TO INCREASE THE EFFECTIVENESS AND CONTROL RELEASE

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Abstract

Extracts from naturally occurred sources, such as plants or microorganisms, have been recently tested for their interesting properties that can be used in food or cosmetic industry.

The aim of this work is to encapsulate microbial compounds, some plant extracts and also model hydrophilic and hydrophobic substances. Chosen components were encapsulated into liposomes.

Particle size and distributions of liposomes were analyzed by dynamic light scattering and their stability by using zeta potential. Spectrophotometric methods were used to measure encapsulation efficiency and to characterize the chosen plant extracts by its amount of total polyphenols, flavonoids and antioxidant activity.

Chosen samples were analyzed for their antimicrobial activity and cytotoxicity. *Serratia marcescens, Escherichia coli* as gram-negative bacterias, *Micrococcus luteus* as gram-positive bacteria and fungal strain (*Candida glabrata*) were used to test antimicrobial activity. Human keratinocytes and mouse melanoma cells were used to cytotoxicity determination.

It has been proved that encapsulation techniques help slowly release the active compounds from plant extracts and transport them to targeted location. Antimicrobial effect was also seen before and after encapsulation into liposomes. Moreover, none of the extracts showed evidence of cytotoxicity.

Keywords: Encapsulation, liposomes, polyphenols, antioxidant activity, antimicrobial activity, cytotoxicity

1. INTRODUCTION

Novel antimicrobial research and its results are very often used to combine with antibiotic therapy. This can be new way how to pursue in order to combat and delay resistance of pathogens. Many research groups are studying the effects of natural antimicrobial substances. These agents can be used as stand-alone or adjunctive therapies.[1] Mainly, phenolic compounds are increasingly attracting attention and in this context, the use of essential oils or other plant extract may exert synergistic antimicrobial activity. [1,2,3] Essentials oils and plant extracts are widely used in food, agricultural, cosmetics and pharmaceutical application. However, there are certain limitations, such as, low water solubility, strong organoleptic flavor and low stability. Using of encapsulation techniques might help increasing these components' chemical stability and solubility. Encapsulation into different type of particles also supports controlled and sustained release which enhances the bioavailability and efficiency against resistant pathogens. [2,3,4] So, the antimicrobial efficiency against microorganism can be enhanced by applying novel drug delivery systems such as nanoparticles or liposomes.[1] Liposomes are spherical lipid vesicles with a bilayered membrane structure consisting of amphiphilic lipid molecules. A lipid membrane surrounding an aqueous cavity enables to encapsulate both hydrophobic and hydrophilic compounds. One of the most commonly used lipids in liposome preparation is phosphotidylcholine where cholesterol is incorporated into the formulation to adjust membrane rigidity and stability. Currently, liposomes are the most widely used antimicrobial drug delivery system, thanks to direct fusing with bacterial membranes, the antimicrobial component of liposomes can be released to the cell membranes or the interior of the bacteria. [5]

2. MATERIALS AND METHODS

2.1. Chemicals

ABTS (2,2'azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)), Gallic acid, (+)-catechin, Folin-Ciocalteu's reagent, cholesterol, soya bean lecithin, nisin and Trolox (6–hydroxy–2,5,7,8–tetramethylchroman)–2–carboxylic acid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other solvents and reagents used in the analysis were of analytical grade.

2.2. Plant extracts and their characterization

Three different types of extract were prepared (water, ethanol and essential oils). Total phenolic and flavonoid contents, antioxidant activity and antimicrobial activity of these extracts were analyzed. Finally, the extracts were used for encapsulation into liposome. The total phenolic content was measured using the Folin–Ciocalteu colorimetric method. Readings were quantified using a standard curve of gallic acid and the results were expressed in mg of gallic acid equivalent (GAE) per g of dry sample. The total flavonoid content was measured by the aluminium chloride colorimetric method. Readings were quantified using a standard curve of catechin and the results were expressed is mg of catechin equivalent (CE) per gof dry sample. Total antioxidant activity was determined by ABTS radical cation decolorization assay and antioxidant activity was calculated as a change in the absorbance. Results were expressed in mg of Trolox equivalent (TE) per gram of dry sample. The measurements were performed using UV-Vis spectrophotometer (Thermo Spectronic Helios^{TM} δ, Thermo Fisher UK Ltd., Hemel Hempstead, UK).

2.3. Microorganisms and their cultivation

The antibacterial activities of all extracts and liposome were determined against bacterial and yeast strains. Gram-positive *Micrococcus luteus* CCM 1569, Gram-negative bacteria *Serratia marcescens* CCM 8587, *Escherichia coli* CCM 7395 and yeast strain *Candida glabrata* CCM 8270 were used. All microorganism used in this study were supplied by the Czech Collection of Microorganisms in Brno. Bacterial cultures were grown in commercial Nutrient Broth medium, for *Candida glabrata* commercial medium YPD Broth was used. Temperature for cultivation of all tested microorganism was 37 °C.

2.4. Determination of antimicrobial activity

Agar diffusion method and broth dilution method were used to determine antimicrobial activity of extracts and liposomes. Mainly the microbroth dilution assay was used. In this method, microorganisms were inoculated into a liquid growth medium in the presence of different concentrations of testing samples. Specifically, 150 µL of 10^6 CFU/mL of microbial culture was added to sterile 96-well microtiter plates followed by 50 µL of sample. The negative control was prepared by adding 150 µL of microbial culture followed by 50 µL of solvent. Absorbance of the wells was measured using ELISA reader (BioTek Instruments GmbH, Bad Friedrichshall, Germany) at 630 nm, before and after 24 hour incubation at 37°C.

2.5. Preparation of liposomes and their characterization

In this work plant extracts were encapsulated into liposome nanoparticles prepared using sonication techniques from soya bean lecithin. Solution of lecithin 16 mg/mL with the addition of cholesterol 2 mg/mL was used. The lipid dispersion was sonicated (80 W, 20 kHz) using ultrasonic homogenizer (BANDELIN electronic GmbH & Co. KG, Berlin, GER) for a few minutes to form liposomes. Size of prepared liposomes and stability of liposomes were analyzed thanks to its zeta potential by dynamic light scattering using colloidal DLS analyzer Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). Encapsulation efficiency was measured spectrophotometrically.

The content of encapsulated polyphenols components was measured before and after encapsulation. The percentage of encapsulation efficiency (EE) was then calculated according to the following equation:

$$EE = \frac{m_t - m_f}{m_t} \cdot 100 \quad (1)$$

where:

m_t - total polyphenols before encapsulation (g)

m_f - free polyphenols after encapsulation (g)

3. RESULTS AND DISCUSSION

3.1. Characterization of plant extracts

In this study content of phenolics and flavonoids in prepared extracts was measured. Total antioxidant activity of tested extracts was determined too. The highest content of total phenolic components was determined in essential oil of clove, in ethanol extract and water water extract of clove and cinnamon (**Figure 1**). Aqueous extract sample of acerola was also characterized. Acerola extract showed even higher amount of total phenolics than tested spices - 23, 87 mg/g.

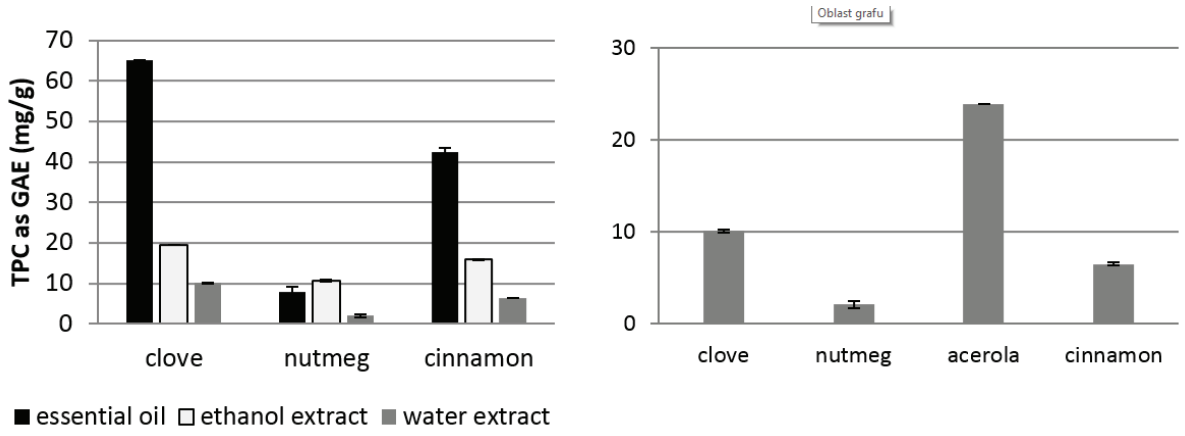

Figure 1 Content of total phenolic components in different type of extracts

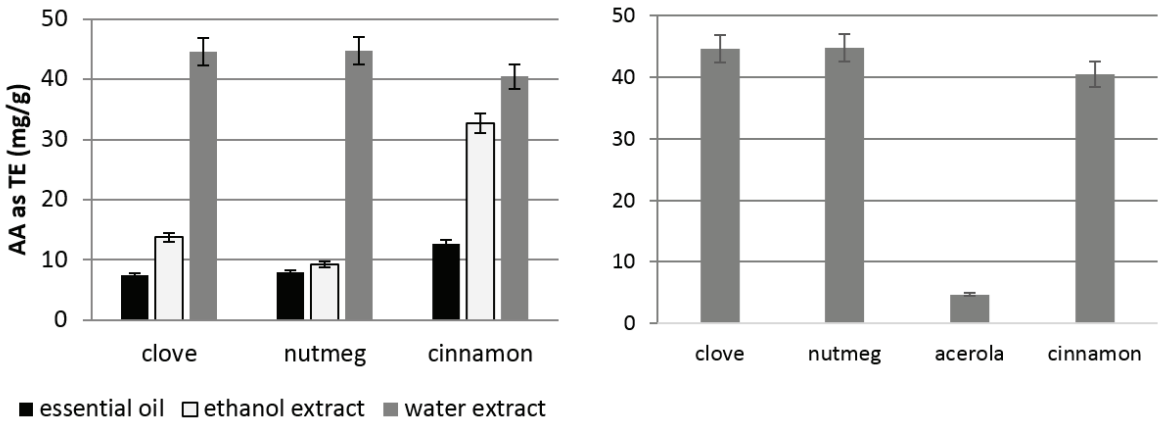

Figure 2 Antioxidant activity of different type of extracts

High antioxidant activity was observed mainly in water extracts (**Figure 2**) and the highest antioxidant activity was determined in nutmeg and clove. It is interesting that extracts of nutmeg had low concentration of total

phenolic components, while exhibited high antioxidant activity. These results indicate that the phenolic components are not a sole source of antioxidant activity of these herbal extracts.

3.2. Encapsulation of plant extracts and determination of particle stability

Three different types of plant extracts were encapsulated into liposomes. Encapsulation was successful for all tested spice extract and encapsulation efficiency was in almost all case higher than 70 % (**Figure 3**).

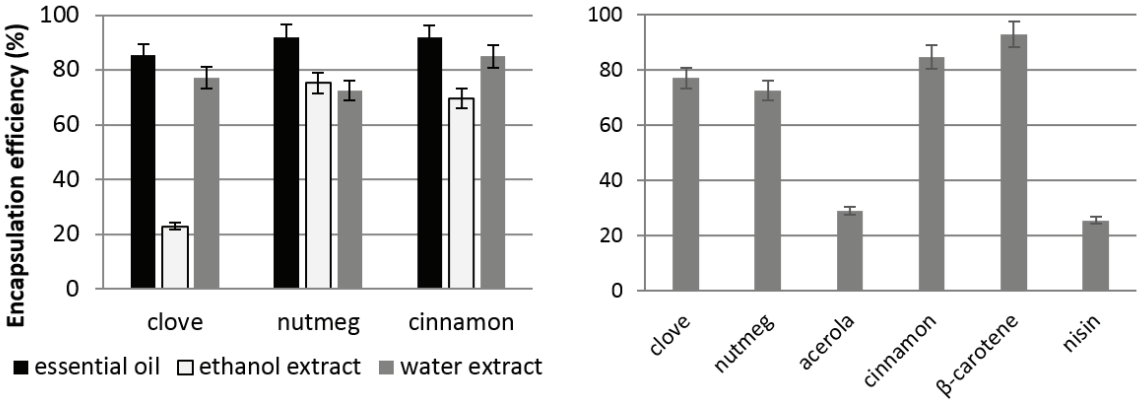

Figure 3 Encapsulation efficiency of different type of extracts

Size of particles was analyzed by dynamic light scattering and the size of liposomes was in ranged from 100 to 320 nm. Stability of the particles was then measured using zeta potential and all prepared particles exhibited very good stability (**Table 1**).

Table 1 Size and stability of liposomes measured by dynamic light scattering and zeta potential

source of encapsulated water extract	star anise	clove	nutmeg	cinnamon	acerola	β-carotene	nisin
Ø size [nm]	172.6 ± 2.1	146.7 ± 0.8	164.2 ± 1.0	159.1 ± 1.9	130.8 ± 1.6	132.3 ± 0.7	106.7 ± 0.9
ZP [mV]	- 49.7 ± 1.1	- 46.9 ± 0.8	- 45.1 ± 1.6	- 43.4 ± 0.6	- 38.9 ± 1.1	- 44.1 ± 1.2	- 42.4 ± 0.9

3.3. Determination of antimicrobial activity

In this study the antimicrobial activity of different types of plant extracts encapsulated into liposomes were measured. The best antimicrobial activity was found in liposomes with essential oils. These particles showed good antimicrobial activity against all tested strains (**Table 2**).

Table 2 Antimicrobial activity of spice extracts-load liposomes

microorganism	*M. luteus*			*S. marcescens*			*C. glabrata*		
type of encapsulated extract	essential oil	ethanol	water	essential oil	ethanol	water	essential oil	ethanol	water
clove	+++	+++	+	++	+++	+	+++	+	+
nutmeg	+++	+++	++	+++	+	+	+	-	+
cinnamon	+++	++	+	++	+	+	+++	-	+

0-35 % growth compared to the control (+++), 36-70 % growth compared to the control (++), 71-90 % growth compared to the control (+) 100 % growth compared to the control (-)

Liposomes with chosen aqueous extract were combined with nisin in specific ration according to their individual antimicrobial activity. Some samples showed synergism when antimicrobial activity was higher than activity of individual nisin sample. Acerola, cinnamon and clove extracts might be the best extracts to be combined with nisin thanks to high antimicrobial activity against *Micrococcus luteus, Escherichia coli and Candida glabrata* (**Table 3**).

Table 3 Antimicrobial activity of combined extracts of nisin and plant extract

microorganism		*M. luteus*				*S. marcescens*				*E. coli*				*C. glabrata*			
concentration [mg/mL]		0.1		20		0.1		10		0.2		50		0.1		10	
nisin	extract																
ratio (nisin:extract)		1:0	0:1	1:1	1:2	1:0	0:1	1:1	1:2	1:0	0:1	1:1	1:2	1:0	0:1	1:1	1:2
clove		-	++	+	+	+	+	-	+	+	-	-	+	-	+	+	+
cinnamon		-	+	-	+	+	+	-	-	+	-	-	-	-	+	+	+
acerola		-	+	+	++	+	+	-	+	+	++	+++	+++	-	+	+	+

0-20 % growth compared to the control (+++), 21-50 % growth compared to the control (++), 51-90 % growth compared to the control (+) 100 % growth compared to the control (-)

3.4. Long-term stability and safety of prepared liposomes

The long-term stability of liposomes containing plant extract was tested in model conditions. The liposomes were also added into different type of cosmetic product (gel and emulsion) and stability of liposomes and stability of cosmetic product during storage were measured. The prepared particles exhibited very good long-term stability in aqueous conditions. Relatively good stability of particles was determined in cosmetic emulsion. Nevertheless, particles stored in the presence of higher concentrations of fat were less stable.

Furthermore, it is necessary to test potential genotoxic and cytotoxic effects before application of prepared particles. Genotoxic effects of extract-loaded liposomes were analyzed by commercial SOS Chromotest kit and it was found, that neither particles show any genotoxic effect. Next, the cytotoxicity of liposomes was assessed by MTT assay on human epidermal keratinocytes (HEK). The MTT assay was used to assess the viability of HEC with different concentration of tested liposomes and it was confirmed that all tested liposomes particles with concentration from 2 to 14 % do not have toxicity effect.

4. CONCLUSION

Liposome particles with encapsulated hydrophilic or hydrophobic antimicrobial plant extract, especially spices, could be used in cosmetics and pharmacy applications as an alternative to antibiotics. Antimicrobial effect together with antioxidant activity of plant extracts even combined with animal origin substances, such as nisin, could be served as a very promising tool for food preservation or other antimicrobial products. Other potential applications of prepared particles are in disinfection or wound healing therapy.

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