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ENCAPSULATION OF EUCALYPTUS LEAVES PHYTOPRODUCTS INTO LIPOSOMAL NANOPARTICLES AND STUDY OF THEIR ANTIBACTERIAL ACTIVITY AGAINST *STAPHYLOCOCCUS AUREUS IN VIVO*

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ABSTRACT

Plant oils are high effective active pharmaceutical ingredients, but the extremely low bioavailability of lipophilic compounds limits their use in drugs. The encapsulation of essential oils into nanoparticles are actively studied. The aim of the research was to prepare liposomal forms of eucalyptus leaves extract and Chlorophyllipt and to study their antibacterial activity. Liposomal forms of Chlorophyllipt and eucalyptus oil extract was prepared using egg phosphatidylcholine and cholesterol by high pressure homogenization and sonication method. The average particle sizes of liposomal Chlorophyllipt and liposomal eucalyptus extract were 156.5 and 210.4 nm, respectively. Active pharmaceutical ingredient encapsulation in liposomal Chlorophyllipt and liposomal eucalyptus extract were at least 85% and at least 90%, respectively. Antibacterial activity of liposomal drugs was studied in white mice with model of staphylococcal infection, initiated by *Staphylococcus aureus* ATTC 209. The use of liposomal eucalyptus oil and liposomal Chlorophyllipt demonstrates no toxicity in mice. Both investigated drugs demonstrated antibacterial activity against *Staphylococcus aureus* in mice. A single administration of the liposomal drugs increased the survival rate up to 30-40% compared with model animals. Double administration of liposomal drugs both in liquid form and in lyophilized form increased the survival rate by at least 70 %. Moreover, in the group of animals treated liposomal eucalyptus oil, the number of surviving animals was 100%, while in a group of liposomal Chlorophyllipt it was 80%.

Keywords: liposomal drug, eucalyptus oil, chlorophyllipt, antibacterial activity, *Staphylococcus aureus*

INTRODUCTION

Nanoproducts, and in particular liposomes, have a wide spectrum of action and are used as a drug delivery system **(Rommasi and Esfandiari, 2021; Shvets *et al*, 2016)**. Currently, the prospects for the use of liposomal drugs are beyond doubt due to the following advantages of this dosage form: protection of body cells from the toxic effect of the active pharmaceutical ingredient (API); prolongation of the API action in the body; changes in pharmacokinetics of the API that increase its pharmacological efficacy; possibility of creating a water-soluble form of lipophilic APIs, thereby increasing their bioavailability etc. **(Grigorieva and Krasnopolsky, 2020; Stefanov *et al*, 1994)**. The liposomal drugs creation is one of the promising areas of modern nanopharmacology, and there are about 50 liposomal products on the pharmaceutical market today. For a number of years, we have conducted research aimed at creating of liposomal water-soluble forms of lipophilic compounds. Liposomal drugs with lipophilic APIs (phosphatidylcholine in "Lipin" **(Grigorieva and Krasnopolsky, 2020; Stefanov *et al*, 1994)**, quercetin in "Lipoflavon" **(Stefanov *et al*, 2006; Grigorieva *et al*, 2016)**, antral in "Lioliv" **(Grigorieva *et al*, 2003)**, manufactured by Biolik, Kharkiv) were obtained and licensed in Ukraine. The development of technologies for a number of liposomal lipophilic APIs, such as docetaxel, curcumin, coenzyme Q10, etc., has been carried out **(Pylypenko *et al*, 2020; Krasnopolsky and Dudnichenko, 2017; Vafadar *et al*, 2020; Zawilska *et al*, 2021; Shakhmaev and Krasnopolskyi, 2012; Shakhmaev *et al*, 2013a)**. It should be noted that the use of APIs extracted from plants (curcumin, quercetin, coenzyme Q10) is promising for drugs creation **(Stefanov *et al*, 2006; Grigorieva *et al*, 2016; Pylypenko *et al*, 2020; Pylypenko *et al*, 2019)**.

Plant essential oils are promising lipophilic APIs, since they have high antibacterial, antifungal, and antiviral activity **(Swamy *et al*, 2016)**. Plant oils contain various aldehydes, terpenes, and phenols, that makes them indispensable to fight pathogens in humans **(Swamy *et al*, 2016)**. Due to the extremely low bioavailability of lipophilic APIs, studies are conducted on the encapsulation of essential oils into nanoparticles: metallic, polymeric, solid lipid nanoparticles, liposomes, etc. For example, the antibacterial activity of nanoemulsions containing eucalyptus oil against *Staphylococcus aureus* was shown *in vitro* **(Sugumar *et al*, 2015; Clavijo-Romero *et al*, 2019)**. Liposomes containing eucalyptus oil demonstrated antibacterial activity against gram-positive and gram-negative bacteria, such as *S. aureus* **(Lin *et al*, 2015; Saporito *et al*, 2017)**, *Streptococcus pyogenes* **(Saporito *et al*, 2016)**, *Escherichia coli*, and *Pseudomonas aeruginosa* **(van Vuuren *et al*, 2010)** *in vitro*. Eucalyptus oil in liposomal form demonstrated an increase in the pharmacological activity compared to free form of API **(van Vuuren *et al*, 2010)**. The antifungal activity of liposomal and polymeric nanoparticles with eucalyptus oil has been shown **(Caetano *et al*, 2022; Moghimipour *et al*, 2012)**. The proven antibacterial activity of eucalyptus oil and its low toxicity to healthy tissues in the body **(Gherasim *et al*, 2021)** make it a promising topical agent for wound healing **(Saporito *et al*, 2016; Alam *et al*, 2018)**. The possibility of using liposomal form of eucalyptus oil in the composition of transdermal gels has been established **(Moghimipour *et al*, 2012; Aziz *et al*, 2019)**. The advantage of liposomal nanoparticles is the possibility of creating injectable dosage forms.

In Ukraine in the 1970s, prof. V.L. Nadtoka has developed a highly effective drug based on eucalyptus leaves extract and chlorophyll, called Chlorophyllipt **(Nadtoka *et al*, 1979)**. Chlorophyllipt is obtained from the leaves of *Eucalyptus globulus* and/or *Eucalyptus viminalis* (Myrtle family) by extraction of APIs, followed by stabilization and purification. The eucalyptus leaves contain an essential oil (up to 3 %), that consists of 1.8-cineol (up to 80 %, M.M. – 154.249), myrtenol and cyclic terpenes (α-pinene and β-pinene), potentiating the action of cineole **(Yakovleva, 2008)**. The developed drug has anti-inflammatory, antiseptic, fungicidal activity. It exhibits antibacterial activity against gram-positive and gram-negative microorganisms, such as *S. aureus*, *E. coli*, mycobacteria, etc. Chlorophyllipt is available in 2 dosage forms for external use, as an oil and an alcohol solution. Chlorophyllip can be introduced into the composition of other pharmaceutical products. Thus, a drug containing phytoproducts (soy phosphatidylcholine, chlorophyllipt, quercetin, curcumin, and vitamin E) was proposed and studied on a model of peptic ulcer in rats (25 mg of diclofenac sodium per 1 kg of body weight), the combined drug prevented the appearance of organic lesions on the stomach mucosa **(Shakhmaev *et al*, 2013a,b)**. Research aimed at finding new approaches to the development and standardization of phytopreparations from essential oil raw materials, in particular from eucalyptus leaves is being carried out **(Zilfikarov, 2008)**.

Thus, the liposomal forms of eucalyptus extract and Chlorophyllipt are of interest for pharmacy. The aim of this work was to prepare liposomal forms of eucalyptus

extract and Chlorophyllipt and to study their antibacterial activity against *S. aureus* ATTC 209 in mice.

MATERIAL AND METHODS

Objects of the research

The liposomal form of Chlorophyllipt and the liposomal form of the total lipophilic extract of *Eucalyptus globulus* leaves were used in the work. The liposomal form of Chlorophyllipt was prepared according to a previously developed method **(Krasnopolsky *et al*, 2006)**. Chlorophyllipt alcohol solution manufactured by Experimental Plant "GNTSLS" (Kharkiv) was used for liposomes preparation.

Obtaining of eucalyptus essential oil

The total extract was obtained from the leaves of *Eucalyptus globulus* by grinding and extraction with organic solvents, ethanol, petroleum ether (boiling point 40-70 °C) and methanol. Eucalyptus oil was extracted either in a thermostat (with stirring) or in a Soxhlet apparatus at the solvent boiling temperature. The extraction was carried out several times. The ratio of crushed leaves to organic solvent was 1:30. The resulting extracts were kept at a temperature of 5-10 °C to precipitate insoluble impurities, which were separated by filtration or centrifugation. The solutions were mixed and concentrated to an oil in a Buchi rotary evaporator. The resulting eucalyptus essential oil was encapsulated in liposomes according to the method **(Krasnopolsky *et al*, 2006)**.

Preparation of liposomal forms

Both liposomal drug samples were prepared using egg phosphatidylcholine (EPC) (90 %) and cholesterol (10 %) by high pressure homogenization and/or sonication method. Given the instability of essential oils under the influence of oxygen, light and temperature, all processes of liposomes preparation were carried out using nitrogen gas atmosphere, limited light and at the lowest possible temperature. The resulting liposome emulsion was filtered using sterilizing filtration and placed into 5 ml vials. The samples were lyophilized to improve product stability. When working with eucalyptus essential oil, the content of volatile components must be taken into account.

Analytical methods for essential oil and liposomal forms studying

Liposomal forms of Chlorophyllipt and eucalyptus essential oil were studied by chromatography in a silica gel thin layer. Lipid components (EPC and cholesterol) and liposomal samples were dissolved in a mixture of chloroform and methanol in a ratio of 2:1. 10 µL of samples were applied onto silica gel plates. A mixture of chloroform, methanol and water was used as the mobile phase in a ratio of 65:25:4. After chromatography, the plates were dried in air (for 10-15 minutes) and treated with iodine vapor. Fractions of EPC and cholesterol were indicated on the chromatograms using standard samples manufactured by Sigma Aldrich.
Samples of essential oils and their liposomal forms were studied by chromatography in a silica gel thin layer. 10 µl samples were applied onto silica gel plates. A mixture of ethyl acetate and toluene in a ratio of 10:90 was used as the mobile phase. After chromatography, the plates were dried in air (for 10-15 minutes), treated with anisaldehyde and heated at 105 °C (for 10 minutes). The main purple-brown spot of cineole was found, corresponding to the standard cineole fraction. The content of 1,8-cineol in eucalyptus essential oil samples was not less than 72%. In the region of 240–320 nm, the sum of phenolic aldehydes (APIs of eucalyptus oil) extracted from eucalyptus leaves has an absorption maximum at 278 ± 2 nm **(Zilfikarov, 2008)**.
The size of liposomes was measured on a Malvern Zetasizer Nano ZS nanosizer (UK) using a semiconductor laser at a wavelength of 375 nm and a temperature of 30 °C.

Characteristics of the test strain

S. aureus ATTC 209 was used in the work as a test strain. The test strain met the following requirements: it formed a hemolysis zone 3-5 mm in size after 19±1 hours of growth on meat-peptone agar, containing 5% human blood, coagulated rabbit plasma within 12±1 hours. The test strain caused necrosis in rabbits within 48-72 hours after intradermally administration of 200 million microbial cells of a daily culture dissolved in 0.2 ml of 0.9 % sodium chloride. To separate from the culture liquid, the resulting culture of the reference bacterial strain was centrifuged for 10-15 minutes at 2000 rpm and washed with a sterile 0. 9% sodium chloride solution.

Model of staphylococcal infection

The experiment was carried out on white mice weighing 20-22 g. The *S. aureus* strain was intraperitoneally administered at a concentration of 1×10^5 bacterial cells per ml of sterile 0.9% sodium chloride solution. The injection volume was 1 ml per mouse, that led to the lethality of at least 70% of the animals after 72 hours. Liposomal forms were administered intravenously at a dose of 2 mg (dissolved in 0.5 ml) twice with an interval of 24 hours. Animals were observed for 10 days after infection. Animals (100 mice) were divided into 10 groups of 10 animals each. The animals were kept under standard conditions in accordance with the regulations of the National Research Council (2010). All procedures were conducted in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986), and the experimental protocol was approved by the local animal ethics committee.

RESULTS

Liposomal Chlorophyllipt was a dark green product, and liposomal eucalyptus extract was a light yellow product. The shelf life of the lyophilized drugs was at least 2 years (at a storage temperature of -5–0 ºC), and the shelf life of the liquid drugs was at least 3 months (at a storage temperature of 2–8 ºC). Both lyophilized liposomal products were readily emulsified in water for injection. In lyophilized samples, APIs encapsulation in liposomes was at least 85% for Chlorophyllipt and at least 90% for essential oil. In liquid samples, APIs encapsulation in liposomes was at least 90% for Chlorophyllipt and at least 93% for essential oil. The average particle size of liposomal Chlorophyllipt was 156.5 nm, and the average particle size of liposomal eucalyptus extract was 210.4 nm.
The results of studying the antibacterial activity of Liposomal Chlorophyllipt and Liposomal extract of *Eucalyptus globulus* in 10 groups of mice are presented in Table 1.

Table 1 Survival and average weight of *S. aureus* infected mice, treated by liposomal drugs

Group of animals	Dose of *S. aureus* (cells per mouse)	Investigated drug	Dose of drug (mg per mouse)	Drug administration mode	Number and percent of surviving animals	Average weight of surviving animals, mg
1 (model)	1×10^5	–	–	–	1/10	20,0
2	1×10^5	LsChL (liquid)	2	Once, 24 hours before infection	3/30	23,6
3	1×10^5	LsEuc (liquid)	2	Once, 24 hours before infection	4/40	24,8
4	1×10^5	LsChL (liquid)	2	Twice, 1 and 24 hours after infection	8/80	27,0
5	1×10^5	LsEuc (liquid)	2	Twice, 1 and 24 hours after infection	10/100	26,8
6 (intact)	–	LsChL (liquid)	2	Twice with an interval of 24 hours	10/100	30,2
7 (intact)	–	LsEuc (liquid)	2	Twice with an interval of 24 hours	10/100	31.4
8	1×10^5	LsChL (lyophilized)	2	Twice, 1 and 24 hours after infection	7/70	26,0
9	1×10^5	LsEuc (lyophilized)	2	Twice, 1 and 24 hours after infection	8/80	26,7
10 (intact)	–	LsChL (lyophilized)	2	Twice with an interval of 24 hours	10/100	30,5

Legend: LsChL – Liposomal Chlorophyllipt; LsEuc – Liposomal extract of *Eucalyptus globulus*

Studies have shown that the liposomal forms of Chlorophyllipt and eucalyptus oil do not affect the survival of uninfected (intact) animals. All mice remained healthy and gained weight during 10 days of observation (groups 6, 7, 10). Thus, the use of liposomal eucalyptus oil and liposomal Chlorophyllipt demonstrates no toxicity in mice, which is consistent with the data available in the literature. For example, it was shown that metal nanoparticles with eucalyptus oil did not cause histological changes in the tissues of the brain, myocardium, pancreas, liver, kidneys, and lungs *in vitro* **(Gherasim *et al*, 2021)**. The effectiveness of eucalyptus oil encapsulated in chitosan nanoparticles against nematodes in mice was on the same level as the free drug, but the encapsulation of oil in nanoparticles reduced toxicity (LD50) by 1.6 times **(Ribeiro *et al*, 2014)**.

Both investigated drugs demonstrated antibacterial activity against *S. aureus* in mice. A single administration of the liposomal drugs (24 hours before infection) increased the survival rate up to 30-40% (groups 2, 3) compared with model animals. Double administration of liposomal drugs (1 hour and 24 hours after infection) both in liquid form (groups 4, 5) and in lyophilized form(groups 8, 9) increased the survival rate by at least 70 %. Moreover, in the group of animals treated liposomal eucalyptus oil (group 5), the number of surviving animals was 100%, while in a group of liposomal Chlorophyllipt (group 4) it was 80%. It may be due to the selective extraction of essential oil with three organic solvents, which led to an increase in content of APIs in the total extract of eucalyptus leaves. The liposomal form of eucalyptus oil can increase the bioavailability of the API. Attention is drawn to the fact that the use of Chlorophyllipt, containing both eucalyptus oil and chlorophyll, does not exceed the antibacterial activity of the liposomal form of eucalyptus oil.

DISCUSSION

Analyzing the literature data, we noted that the compositions of nanoparticles with eucalyptus oil significantly differ (Table 2), which causes different physicochemical properties of the products. For example, the particle size of the liposomal forms of eucalyptus oil varied from 50 to 900 nm, the degree of inclusion was not less than 70%. Comparing the results obtained in this work with properties of eucalyptus oil nanoforms presented in the literature (Table 2), the samples demonstrated satisfactory sizes, size uniformity, and the level of APIs encapsulation into liposomes.

Table 2 Formulation, physicochemical and pharmacological properties of eucalyptus oil nanoparticles

API	Nanoparticle formulation	Particle size	Level of encapsulation	Zeta potential	Pharmacological activity	Model	Ref.
Eucalyptus citriodora oil	Liposomes of EPC, cholesterol, polyvinyl-lpyrrolidone (20:5:1)	63.9 nm	N/A	-25 mV	Antimicrobial activity against *S. aureus*	*In vitro*	Lin *et al*, 2015
Eucalyptus globulus oil	Chitosan nanoemulsion	9.4 nm	N/A	N/A	Antimicrobial activity against *S. aureus*	*In vitro*	Sugumar *et al*, 2015
Eucalyptus globulus oil	Lipid nanoparticles of lecithin, cocoa butter, olive oil (2 : 2 : 1)	50–60 nm	101.74 ± 9.06 %	-22.07 ± 0.29 mV	Antimicrobial activity against *S. aureus* ATCC 6538, *Streptococcus pyogenes* ATCC 19615	*In vitro*	Saporito *et al*, 2017
					Wound healing activity	In rats (externally)	
Eucalyptus globulus oil	Chitosan-coated liposome of diastearoyl phosphatidyl choline and diastearoyl phosphatidyl ethanolamine	885 ± 119 nm	69.2 %	13.0 ± 3.75 mV	Antimicrobial activity against *Candida albicans* ATCC 10231, *E. coli* ATCC 8739, *Pseudomonas aeruginosa* NCTC 9027 and *S. aureus* ATCC 6538	*In vitro*	van Vuuren *et al*, 2010
Eucalyptus globulus oil	Polylactic acid-coated iron oxide nanoparticles (Fe_3O_4)	7.5 ± 2.5 nm	N/A	N/A	Antimicrobial activity against *E. coli* ATCC 15224 and *S. aureus* ATCC 25923	*In vitro*	Gherasim *et al*, 2021
Eucalyptus citriodora oil	Nanoemulsions of Tween-85 and Transcutol (1:1)	32.45 ± 2.84 nm	N/A	-34.25 mV	Would healing activity	In rat (orally)	Alam *et al*, 2018
Eucalyptus citriodora oil	Chitosan nanoemulsion	232 nm	N/A	N/A	Antiparasitic activity against *Haemonchus contortus*	*In vitro* and in mice	Ribeiro *et al*, 2014
Eucalyptus citriodora, Eucalyptus camaldulensis and Eucalyptus grandis oil	Poly(ε-caprolactone) nanoparticles	402.13 nm, 275.33 nm, 328.5 nm, respectively	N/A	-11.8 mV, -9.24 mV -6.76 mV, respectively	Antifungal activity against *Hemileia vastatrix*	*In vitro*	Caetano *et al*, 2022
Eucalyptus camaldulensis oil	Soya lecithin and cholesterol (1:1)	156.33 ± 1.55 nm	95.0 ± 0.57 %	N/A	Antifungal activity against *Microsporum canis* PTCC 5069, *M. gypseum* PTCC 5070, *Trichophyton rubrum* PTCC. 5143 and *T. verrucosum* PTCC 5056	*In vitro*	Moghimipour *et al*, 2012

The mechanism of the antibacterial activity of eucalyptus oil has not yet been established. Of interest is the suggestion of Lin et al. regarding the effectiveness of the liposomal form of eucalyptus oil against *S. aureus* **(Lin *et al*, 2015)**. Lin et al. showed that liposomal eucalyptus oil inhibited the growth of *S. aureus*, but not the growth of E. *coli*. However, the free form of eucalyptus oil demonstrated an antibacterial effect on both strains. The authors suggested that, unlike *E. coli*, *S. aureus* secretes enzymes that destroy the liposomal membrane, and the encapsulated eucalyptus oil comes out of the nanoparticle and exhibits an antibacterial effect **(Lin *et al*, 2015)**. Meanwhile, other authors noted the antibacterial activity of liposomal and nanoparticles of metals with eucalyptus oil against *E. coli* **(van Vuuren *et al*, 2010; Gherasim *et al*, 2021)**.

The eucalyptus oil encapsulation into liposomal membtane allows expanding the pharmacological properties of phytoproducts. It has now been established that liposomal forms of eucalyptus oil have a pronounced antibacterial and antifungal effect **(Lin *et al*, 2015; van Vuuren *et al*, 2010; Saporito *et al*, 2017; Moghimipour *et al*, 2012; Gupta *et al*, 2022; Tang and Ge, 2017).** It has been shown that lipid nanoparticles with eucalyptus essential oil have antimicrobial activity and can be successfully used for wound healing **(Alam *et al*, 2018; Saporito *et al*, 2017)**. Previously, we studied the pharmacological activity of the liposomal Chlorophyllipt in rats with two models of periodontitis caused by endotoxin injection or using homogenized food **(Krasnopolsky *et al*, 2011)**. The use of the liposomal Chlorophyllipt demonstrated periodontal-protective activity, which, in our opinion, is due to the antioxidant, antihypoxic, antibacterial and membrane-stabilizing effect of the liposomal Chlorophyllipt.

In our opinion, liposomes *per se* have a pharmacological effect, for example, the commercial drug Lipin (lyophilized liposomes from EPC) has a membrane-protective and antioxidant activity **(Grigorieva and Krasnopolsky, 2020; Stefanov *et al*, 1994)**. Van Vuuren et al. demonstrated the synergism of the antibacterial effect of the API and the lipid part in the liposomal eucalyptus oil based on distearoylphosphatidylcholine and distearoylphosphatidylethanolamine. It was shown that eucalyptus oil and empty liposomes have an antimicrobial effect on *Candida albicans*, *E. coli*, *Pseudomonas aeruginosa*, *S. aureus*, while the effectiveness of the liposomal eucalyptus oil exceeds both drugs separately **(van Vuuren *et al*, 2010)**.

CONCLUSION

Liposomal form of eucalyptus oil extracted from *Eucalyptus globulus* leaves and liposomal form of Chlorophyllipt with satisfactory physicochemical properties were prepared. The antibacterial activity of the liposomal form of eucalyptus oil and the liposomal form of Chlorophyllipt against *S. aureus* was studied *in vivo* in mice. Both investigeted drugs demonstrated efficacy, and in the group of mice

treated with the liposomal eucalyptus extract, the lethality was lower than in the group of liposomal Chlorophyllipt. Liposomal forms of the eucalyptus extract and Chlorophyllipt had no toxic effect on intact mice. Obtaining of liposomal form of eucalyptus oil based on natural phospholipids is a promising strategy for creating medicinal, preventive and cosmetic products.

Conflict of interest: The authors declare that there is no conflict of interest.

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