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Determination of Antimicrobial Activity of Antibiotics Encapsulated in Liposomes

Enkhtaivan Erdene[1,2], Enkhjargal Dorjbal[3], Oidov Baatarkhuu[4], Ariunsanaa Byambaa[1]

[1]Department of Microbiology, Infection Prevention and Control, School of Biomedicine, Mongolian National University of Medical Sciences, Ulaanbaatar, Mongolia;
[2]Etugen University, Ulaanbaatar, Mongolia;
[3] School of Pharmacy, Mongolian National University of Medical Sciences, Ulaanbaatar, Mongolia;
[4]Department of Infectious Diseases, School of Medicine, Mongolian National University of Medical Sciences, Ulaanbaatar, Mongolia.

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Corresponding Author:
Ariunsanaa Byambaa (M.D. Ph.D., Prof.)
Department of Microbiology, Infection Prevention and Control, School of Biomedicine,
Mongolian National University of Medical Sciences, Ulaanbaatar 14210, Mongolia.
Tel: +976-99829755
E-mail: ariunsanaa.b@mnums.edu.mn
ORCID: https://orcid.org/0000-0001-8974-2892

Co-Corresponding Author:
Oidov Baatarkhuu (M.D., Ph.D., Sc.D., Prof.)
Department of Infectious Diseases, School of Medicine, Mongolian National University of Medical Sciences, Ulaanbaatar 14210, Mongolia.
Tel: +976-99094624,
E-mail: baatarkhuu@mnums.edu.mn
ORCID: https://orcid.org/0000-0002-0769-3693

Objective: Antibiotic-resistant infections caused by bacteria are already a serious concern for humanity, and by 2050, they are most likely to overtake all other causes of death. One of the most frequent causes of skin infections is methicillin-resistant *Staphylococcus aureus* (MRSA), whose resistance to most medications makes treatment challenging. This study aimed to determine the antibacterial activity of liposome-encapsulated doxycycline compared to free doxycycline. **Methods:** This study was conducted using an experimental design. Our study isolated and purified the phospholipid (PL) fraction from egg yolk. Antibiotics were encapsulated using the freeze-thaw process, and phospholipids were extracted via intermittent evaporation. Finally, the liposome's minimum inhibitory concentration (MIC) was effective against MRSA.
Results: Phospholipid fraction isolated from egg yolks with 32% extraction yield of phosphatidylcholine. The thin phase chromatography fraction was phospholipids with a retention factor (Rf) of 0.39. The effectiveness of liposomal doxycycline hyclate over free doxycycline was demonstrated by *in vitro* tests. **Conclusion:** The results showed that a liposome containing phosphatidylcholine could be a tremendous topical antimicrobial construct for treating MRSA infections.

Keywords: Phospholipids, Liposome, Antibiotic, Nanoparticle, MRSA

Introduction

Antibiotic-resistant bacterial infections are already a serious concern for the entire human population and are predicted to overtake heart disease as the leading cause of mortality by 2050 [1]. First, common germs must be combined to form a biofilm to prevent antibiotics from penetrating infection-causing bacteria. Second, the incorrect usage of antibiotics causes the rise of novel types of bacteria that are resistant to antibiotics. Third, other causes exist, including halting experimental research to find new antibiotics [2].

Pharmaceuticals called antimicrobials are essential for treating bacterial infections. However, antibiotics have, for decades, been abused in hospitals, farming, and animal production settings, leading to significant

selection pressure on bacterial species . The result has been the creation and spread of antimicrobial-resistant strains in humans, animals, and the environment, which has led to the escalation of the antibiotic resistance crisis, one of the top significant risks to global public health in the twenty-first century [4].

It is the focus of the health sector in every country, regardless of the income level of the population, because the emergence of bacterial resistance due to the mutation of infectious disease-causing bacteria and inappropriate use of antibiotics increases the time and cost of treating infectious diseases and in some cases, leads to increased mortality. Additionally, bacterial resistance harms the environment, the economy, food safety, and public health. Active drug transport systems are required to get to the target molecules since more and more bacterial illnesses are resistant to antibiotics. Numerous studies indicate that nano/microcarriers are crucial to active transport [5].

A way of creating active pharmaceutical components with identical tiny particles at the molecular level is called nanotechnology, which is entering the pharmaceutical sector. In drug therapy, it is crucial to maintain a specific blood concentration of any medicine for an extended period of time. Conventional medicine, tablets, and capsules, is taken multiple times daily to maintain a consistent blood concentration in chronic and acute disorders [6].

Drug toxicity or ineffectiveness results from medication concentrations in the blood exceeding therapeutic levels as delivery frequency rises. In an effort to address these issues, the manufacture of nano- and micro-pharmaceuticals based on the process of encapsulating biologically active chemicals on polymer-carriers has received a lot of attention recently [7].

Broad-spectrum antibiotic-resistant bacteria have sharply increased, and among Gram-positive bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as one of the most prevalent and severe causes. Vancomycin, linezolid, daptomycin, tigecycline, and telavancin are some antibiotics frequently used to treat skin, soft tissue, and surgical infections caused by MRSA [8].

With 64.41 antibiotics (units per day) incidences in 1000 inhabitants in 2015 [9], Mongolia has the highest antibiotic consumption worldwide. Broadly-spectrum antibiotic-resistant bacteria have sharply grown; among Gram-positive bacteria, methicillin-resistant *Staphylococcus aureus* has emerged as one of the most prevalent and harmful causes. As of 2018, over 11,000 Americans per year pass away from MRSA infection [10].

Several labs have effectively used liposomes as a model system in their research to reflect various features of cell surfaces and bio-membranes [11]. Since Aleika Bendham (Bangyam A.D) first presented this concept in 1965, interest in using liposomes as drug carriers and delivery agents has multiplied, and they are now the most widely used nanoparticle (NP) drug delivery systems that have been given clinical approval [12]. Firstly, it has to do with the simplicity of manufacture. Secondly, the versatility of medications and molecules that can be covered regardless of hydrophobicity, charge, size, and other physicochemical characteristics; thirdly, biocompatibility. Liposomal drug delivery systems have been authorized for use in clinical settings since the FDA approved Doxil® (a liposomal version of the anticancer medication doxorubicin) in 1995 [13].

One of the most urgent issues in medicine is reducing bacterial resistance. Recently, several experimental investigations have been carried out to use liposomal and other nanoparticles to transport antibiotics and medicines to target cells. In the medical industry, phospholipid-based nanocarriers are frequently utilized. This has the benefit of increasing the encapsulated chemicals' bioavailability and enabling their manipulation to deliver specific amounts to target cells [14].

Liposomes are used in pharmaceutical research and show promise in protecting against various enzymes, delivering drugs inside target cells, increasing bioavailability, and protecting active pharmaceutical ingredients from interacting with other substances [15].

Enerelt Urnukhsaikhan, et al. researched "Antibacterial activity and characteristics of silver nanoparticles biosynthesized from Carduus crispus" in 2021 [16]. The increased prevalence of antibiotic-resistant bacteria, such as MRSA, necessitates liposomes to transport antibiotics that selectively kill bacteria. Therefore, better therapies need to be developed to reduce its side effects. New, safe, and effective topical liposomal doxycycline is one solution.

The liposome has been used as a drug delivery vehicle for decades. Biocompatible lipid materials are used to make these drugs; hydrophilic and lipophilic drugs can be delivered with them; bacterial cell membranes can be fused with them; surface modification is simple and straightforward ; and the increased permeability and maintenance effect allows the drug to concentrate at the target site [18].

To produce scientific evidence, the research team will determine how to characterize liposomes' basic raw materials, localize Gladkowski et al.'s (2012) [19] method and protocol, and develop a new treatment strategy against strains of bacteria resistant to multiple drugs.

In our study, the phospholipid, the main raw material of liposomes, was isolated from egg yolk, and doxycycline against MRSA was encapsulated by a liposome isolated from our study. Our study was conducted first in Mongolia and first developed a new experimental protocol.

One of the many processes limiting antibiotic resistance is the active transfer of antibiotics to pathogenic bacteria without affecting healthy cells. The aim of this study was to determine the antibacterial activity of liposome-encapsulated doxycycline compared to free doxycycline.

Materials and Methods

This study was conducted using an experimental design. We used a randomized controlled trial sample design to extract phospholipids as liposomes from 95 grams of egg yolks. Antibiotic-loaded liposomes were subjected to three experiments. To confirm liposome development, TM-1000 tabletop scanning electron microscopy (SEM) was used. Each sample was diluted to 10^{10} degrees to determine its antibacterial activity. We compared the control group to those receiving free doxycycline in the study.

Cholesterol, doxycycline hyclate, and sodium chloride were purchased from Sigma Aldrich (USA). Blood agar, Mueller Hinton (MH) broth, methanol, chloroform, and acetone were obtained from Merck (Germany). We carried out research work in Mongolia between 2018 and 2022 with the support of the Mongolian National University of Medical Sciences, School of Biomedicine, Microbiology Laboratory, School of Pharmacy, Pharmaceutical Chemistry Laboratory, and Mongolians Academy of Sciences, Institute of Chemistry and Chemical Technology.

Bacterial strains

S. aureus ATCC 25923 and MRSA ATCC 2758 were used as a control strain. Bacteria were stored at -80°C in MH broth supplemented with 20% (v/v) glycerol.

Antibiotics susceptibility testing

The disk diffusion method was used to determine the antibiotic susceptibility of Muller Hinton Agar (Difco, Franklin Lakes, NJ, USA). The observed result was compared with the criteria recommended by the Clinical and Laboratory Standards Institute M100-S27(CLSI M100-S27) guidelines and interpreted.

Preparation of liposome

The Gladkowski et al. (2012) method was used in an experiment to isolate phospholipids from egg yolks. 95 g of egg yolk was combined with 100 ml of acetone and stirred with a magnetic stirrer for 10 minutes. After being evenly split into vials, followed by sedimentation in a centrifuge for 10 minutes at 3000 rpm. After the sediment was separated and dried, it was extracted using a 2:1 mixture of chloroform and ethanol; then, it was thoroughly agitated for three hours at 150 min. Filter the resulting solution in the next. To separate the phospholipid fraction, the filtrate was concentrated and then distilled.

Thin-phase chromatography was used to determine phospholipids. The retention factor (Rf) value and color intensity were compared to those of the standardized sample solution to ascertain the findings.

$$\textbf{Rf calculator } \textit{B.Rf} = \frac{\textit{a}(\text{The path taken by the mixture})}{\textit{b}(\text{The path traveled by the solvent})}$$

Determination of fatty acids by gas chromatography

Fatty acid profiling by gas chromatography (GC) was carried out in a Finnigan Focus GC apparatus (Thermos Fisher Scientific, Waltham, MA, USA) equipped with a flame ionization detector and a VF-5 ms column (60 m × 0.25 mm × 0.25 μm). The injection volume was 1 μl (derivatized lipid extracts containing the IS and FAME mixture). The injector and detector temperatures were maintained at 230°C.

The oven temperature was set at 60°C for 1 min, then increased five °C/min to 250°C, three °C/min to reach 310°C and held for 15 min.

By serially evaporating phospholipids, liposomes were created. The "freeze-drying" process was used for antibiotic capsulation.

Antimicrobial susceptibility test

The minimal inhibitory concentration of free doxycycline and liposome-encapsulated doxycycline was evaluated using a liquid growth inhibition assay in 96-well microtiter plates, as recommended by the CLSI guideline. MIC was considered the lowest concentration of antibiotic that completely inhibited the visible growth of bacteria after 18-20 hours of incubation at 35-37°C.

Scanning electron microscopy

In our investigation, a scanning electron microscopy (SEM) TM-1000 tabletop microscope was used to carry out this experiment. Working conditions for instruments: A 15-kV voltage accelerator, solid state backscattered electron detectors with 20–10.000X detector magnification (digital zoom: 4X), maximum sample size 70 mm in diameter, lower sample height 20 mm, and moving size 15 mm by 18 mm.

Statistical Analysis

The data were analyzed using IBMSSPS version 22.0 software. The sacred zone, which assessed the antibacterial efficacy of drugs, was measured using the central and standard deviation (mean±SD). A Cochran's test was employed to evaluate sensitivity in relation to CLSI guideline criteria and between-group differences. Significant differences were detected between groups ($p<0.05$) using.

Ethics approval and consent to participate

The IRB Committee of the Mongolian National University of Medical Sciences approved the study protocol№2021/3-07 (approved on 2021.06.04).

Results

The results were considered, as were those obtained for the phospholipid fraction isolated and purified by egg yolk. Using acetone to precipitate and wash the phospholipids yielded the phospholipid fraction isolated from phosphatidylcholine, which was 32%. Thin phase chromatography fraction containing phospholipids had a retention factor of 0.39. Gas chromatography was used for fatty acids in phospholipids (Figure 1).

Table 1. Fatty acid composition of phospholipid fractions

Compound Name	Area	Area %	Conc %
$C_{4:0}$	4025	0.822	5.8
$C_{6:0}$	7654	1.5632	2.2
$C_{8:0}$	4349	0.8882	3.6
$C_{10:0}$	1621	0.3311	5.0
$C_{11:0}$	1856	0.3790	5.0
$C_{14:0}$	1929	0.3941	4.4
$C_{14:1}$	3783	0.7726	3.7
$C_{15:0}$	3102	0.6334	3.8
$C_{15:1}$	1115	0.2278	1.5
$C_{16:0}$	131946	26.948	2.9
$C_{17:0}$	1530	0.3126	14.9
$C_{17:1}$	1665	0.3400	2.1
$C_{18:0}$	101192	20.6669	7.1
$C_{18:1cis}$ /$C_{18:1tra}$	57630	11.7700	14.9
$C_{18:2\ cis\ 9.12}$/C_{18}	2787	0.5691	2.1
$C_{18:3(6)}$	2265	0.4625	7.1
$C_{18:3(3)}$	1505	0.3074	7.2
$C_{20:0}$	4292	0.8765	6.6
Total SFA (%)			59.3
Total MUFA (%)			15.5
Total PUFA (%)			25.2

A total of 21 types of saturated and unsaturated fatty acids were identified.

In general, saturated fatty acids (SFA) account for 59.3% of egg yolk PL, while monounsaturated fatty acids (MUFA) 15.5% and polyunsaturated fatty acids (PUFA) can reach up to 25.2%. Oleic acid ($C_{18:0}$) 7.1% and palmitic acid ($C_{16:0}$)2.9% are examples of saturated fatty acids (Table 1).

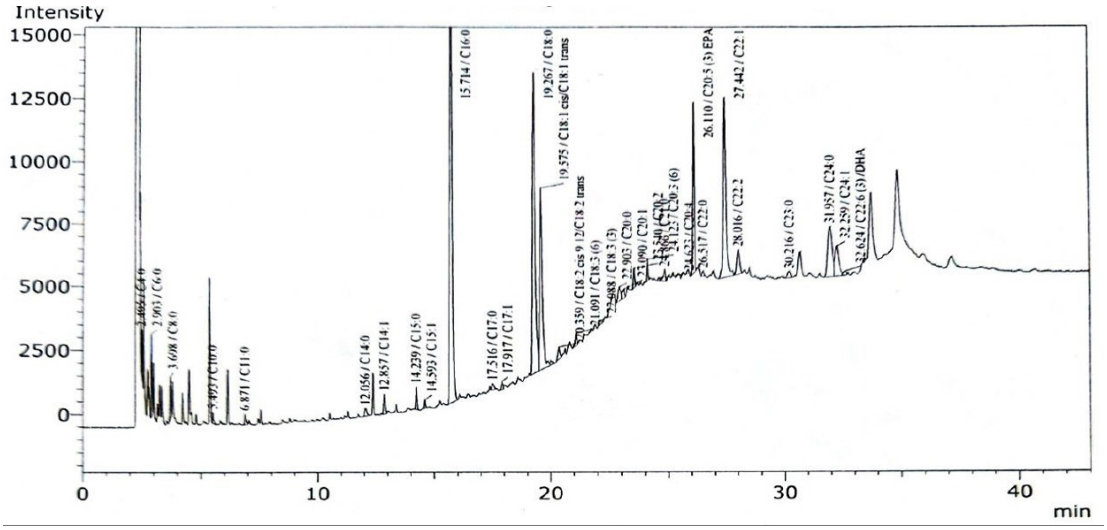

Figure 1. Gas chromatography (GC) measured results

The formation of antibiotic encapsulated liposomes observed under electron microscopy after a layer composed of phospholipids was developed by periodic evaporation (Figure 2).

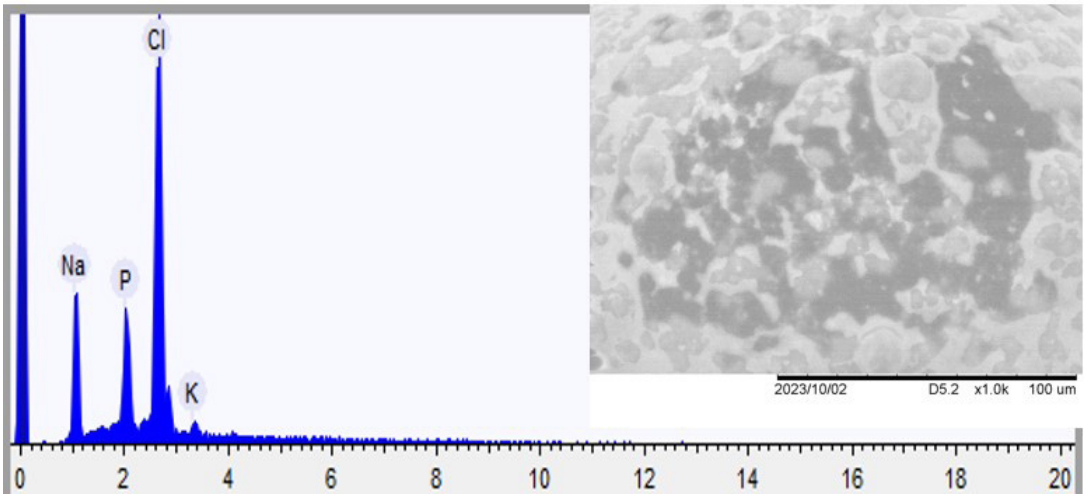

Figure 2. Scanning electron microscope images of liposomes.

Figure 2 shows that areas highlighted in light confirm that electron-dense sparse expression resulted in liposome formation.

In determining the state of liposome formation, it comprised 25.7% sodium, 13.2% phosphorus, 58.8% chlorine, and 2.2% potassium (Figure 2).

Table 2. Antibiotic susceptibility results

Antibiotic	Inhibition Distance Zones /mm/	CLSI guidelines (CLSI M100-S27) /mm/	Antibiotic sensitivity
Azithromycin	7	≤13-18≤	R
Clarithromycin	7	≤13-18≤	R
Doxycycline	18	≤12-16≤	S
Liposome-encapsulated			
Liposome-encapsulated doxycycline	18	≤12-16≤	S
Free liposomal	0	0	R

Abbreviations: S, sensitive; R, resistant;

Table 2 shows antibiotic sensitivity using the disk diffusion method. Doxycycline (20±2) was susceptible (p≤0.05) and developed 18 mm inhibitory distance zones. In contrast, azithromycin (7.3±1.2) and clarithromycin (7.3±1.2) were acquired resistance. The results showed that liposome-encapsulated doxycycline (20±2) created the 18 mm inhibition distance zone. Based on Table 2, free liposome has no antibacterial activity, indicating that only liposomes can be used to deliver drugs.

Table 3. Minimum inhibitory concentration for free and liposomal formulations

Microdilutions	*S. aureus*		MRSA	
	Free doxy	Liposome of doxy	Free doxy	Liposome of doxy
10^1	1	1	1	1
10^2	1	1	1	1
10^3	1	1	1	1
10^4	1	1	1	1
10^5	1	1	1	1
10^6	1	1	0	1
10^7	0	1	0	0
10^8	0	0	0	0
10^9	0	0	0	0
10^{10}	0	0	0	0
P - value	0.011		0.01	

Results observed by the microdilution method showed that the minimum inhibitory dose of free doxycycline and liposome-encapsulated doxycycline. *S. aureus* and MRSA antibiotics were determined by dilutions up to 10^{10}, but the total *S. aureus* could not be inhibited at dilutions of 10^7 or more. However, MRSA is not inhibited at dilutions of 10^5 or higher. Therefore, the standard strain *Staphylococcus aureus* ATCC 25923 was eight mcg/ml and four mcg/ml, respectively. In contrast, in standard strain MRSA, the free doxycycline was 32 mcg/ml, while its liposome-encapsulated doxycycline was 16 mcg/ml(p≤0.05). Liposome-encapsulated doxycycline was more effective in MRSA (p≤0.05) (Table 3). As shown in Table 3, the low dose of antibiotic encapsulated by liposome had high antibacterial activity compared with liposome-free and higher doses. Liposome-encapsulated doxycycline inhibited 40% of MRSA (p≤0.05) and 60% of *S. aureus* (p≤0.05). It is also more active against *S. aureus* than MRSA. (p=0.01).

Discussion

Liposomes have several desirable characteristics, including a lipid bilayer shape that mimics bacterial membranes and facilitates the fusing of the two. As a result, an encapsulated antibiotic can be released for a longer time around or in the interior of a bacterial cell [20], increasing local drug concentrations.

The use of liposomal antibiotics has several benefits, such as reducing the emergence of drug resistance, lowering the dose of the antimicrobial agent, improving drug penetration, lowering drug toxicity, and releasing a significant amount of the drug locally around the bacteria, resulting in an antibacterial effect.

We followed the procedures of Ariunsanaa B.'s (1999) study from the dissertation [21], which was a study to extract phospholipids and install antibiotics. This work obtained Single-layered closed-ring liposome structures due to the "freeze-thawing" process. Our research findings also supported the discovery of several asymmetric liposomes by electron microscopy.

For our experiment, doxycycline was added to liposomes, and the results were similar to those of Hajiahmadi F et al. (2019) investigation; liposomal vancomycin's inhibitory dosage performed better than free vancomycin [22].

According to a study by Abdelkader H et al (2012), using liposomal form led to continuous antibiotic release.

The liposomal version of antibiotics continuously releases antibiotics with long-lasting properties, allowing the MRSA strain to come into contact with antibiotics during this time and strengthening the bactericidal effect [23].

In general, the difference between the free drugs and the entrapment drugs in the upper doses has yet to be discovered because using a high dose of antibiotics leads to the destruction of bacteria. However, the problem above has been solved using

the low-dose liposomal form [24].

Compared to free doxycycline, employing liposomal form allows antibiotics to enter eukaryotic cells more effectively, killing all intracellular and extracellular MRSA. According to our findings, doxycycline has antibacterial activity that has significantly changed from its free form to a successful (16 mm) liposome-encapsulated test.

When tested using the microdilution method, doxycycline dilution as 10^5 resulted in the lowest bacterial inhibition dose, which determined the significance of our research work.

According to the study by Liu Y et al. (2019), Nanotechnology-based antimicrobial agents and biofilms have excellent efficacy and minimal toxicity, which is crucial for therapeutic use. To accomplish this, an antibacterial peptide model, antibiotics, and a method for successfully incorporating azithromycin into liposomes were devised [25].

These research findings, which are consistent with ours, demonstrate that liposomes deliver their medicine to target cells in a stable manner without degrading it. Nevertheless, the majority of investigations have used phospholipids.

Our study shows that a modest growth inhibition dose can effectively suppress MRSA or multidrug-resistant bacteria. This finding has the potential to address several urgent issues facing the healthcare sector and localize drug encapsulating technology.

The results under investigation by Franze S et al. (2018) and Stark B. et al. (2010) are similar to our study.

The experiment used liposome-encapsulated doxycycline. In determining the susceptibility of S. aureus and MRSA strains, liposome-encapsulated doxycycline worked at a dose twofold lower than that of free doxycycline, indicating that it is effective in antibacterial activity.

We will continue our research to improve the yield of phospholipids. Additionally, we plan to determine the formation and size of liposomes and study the possibility of encapsulating the new-generation antibiotics daptomycin and linezolid to evaluate their activity against other pathogens.

Limitations of the study

Financial limitations are detrimental to research. The most difficult aspect was using rare and advanced equipment, such as SEM, TEM, high-performance layer chromatography (HPLC), the Zeta (ζ) potential technique, and dynamic light scattering (DLS). When separating liposomes from raw materials, it is essential to determine the external characteristics of liposome formation by fatty acids. It is also necessary to check the dosage of the antibiotic after encapsulating it in liposomes.

Conclusion

Our study showed that 32 percent of the egg yolk's phosphatidylcholine output was phosphatidylcholine. By thin-phase chromatography, the fraction containing phospholipids has a retention factor of 0.39. Microdilution's results showed that Gram-positive bacteria like *Staphylococcus aureus* and MRSA revealed the efficiency of 2-fold lower dosages of liposome-encapsulated doxycycline.

The primary raw material for this study was phosphatidylcholine, which we separated from egg yolk according to the protocol and determined the yield, properties, and fatty acids. The results of this study further developed that egg yolk can be used to extract liposomes in desired quantities.

Our findings have demonstrated that liposome-capsulated doxycycline inhibited MRSA. The results of our study are evidence-based methodology and technology, which is used for drug delivery systems in the pharmaceutical industry and needed to solve the problem of antibiotic resistance in the world, including Mongolia. Based on the results of international studies, we have established a modified methodology and experimental protocol and tested it first in our country.

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Conflict of Interest

The authors have no competing interests to declare.

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