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Research into Antibacterial Effects of Polysaccharide-Based Microparticles

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Abstract

Background: Due to worldwide increases in the prevalence of antibiotic-resistant bacteria, it is necessary to develop an active drug delivery system that can enable therapeutics to reach their molecular targets. Maintaining the concentration of any drug in the blood at a certain level for a long time is critical in the practice of drug therapy. With the increased frequency of drug use, the blood concentration of drugs exceeds the therapeutic level, leading to toxicity or ineffectiveness. To solve these problems, in recent years, much attention has been given to developing micro/nano preparations by encapsulating biologically active compounds on polymeric carriers. Therefore, we aimed to extract pectin from sea buckthorn peel, prepare microcapsules containing antibiotics, and determine their physical and chemical properties. **Methods:** Wastes were separated from sea buckthorn under "Medical raw materials Dry fruit of *Hippophae rhamnoides* MNS 5225:2002". Pectin was isolated from sea buckthorn waste according to the "method for determination of pectins MNS3080:1981" standard. The degree of esterification was determined according to ISO 7623:2016. Antibiotic encapsulation with coacervates and water-based emulsions was performed. Antibiotic sensitivity was determined by microdilution according to the Clinical Laboratory Standard Institute (MT100-S27) method. The results were determined between standard strains of *Staphylococcus aureus* ATCC 29213 and MRSA ATCC 2758 at different dilution concentrations. **Result:** Pectin is a brown powder with a sour taste and no odor. There was 71.4% esterification of pectin, 8.9% yield, 1.3% free carboxyl group, 3.2% methylated carboxyl group, 4.5% total carboxyl group, 3.5% ash, and 0.1% nitrogen. A study of the antibacterial activity of pectin containing doxycycline hyclate found that the inhibition of bacterial growth was 0.8 times less than that of pure pectin. It was 1 time less than that

of doxycycline alone, and 33 times smaller than that of wontaxime when compared to pure pectin. Pectin containing doxycycline hyclate inhibited MRSA growth at a concentration 6 times lower than pure pectin. This was 2 times lower than doxycycline alone, and 8 times lower than wontaxime. **Conclusion:** Pectin yields 1.3 after 60 minutes of separation at a sediment concentration ratio of 1:1.15 and pH = 2. Pectin itself is antibacterial against MRSA.

Keywords

Pectin, Microparticles, Antibacterial, MRSA, Doxycycline Hyclate

1. Introduction

Globally, the occurrence of antibiotic-resistant bacterial infections is increasing, which calls for an active transport mechanism for drugs to enable them to reach their molecular targets [1] [2] [3] [4]. The results of many studies show that nano/micro carriers play a crucial role in active transport mechanisms [5].

Nanotechnology, which is an emerging component of the pharmaceutical industry, is a method of formulating active pharmaceutical ingredients using small particles. Maintaining the blood concentration of any drug at a certain level for a long time is important in the practice of drug therapy. To maintain a constant blood concentration, tablets, and capsules are taken several times a day in the treatment of chronic and acute diseases. As the frequency of drug use increases, blood drug concentrations exceed therapeutic levels, resulting in toxicity or ineffectiveness. To solve these problems, in recent years, much attention has been given to the production of nano/micro pharmaceuticals based on the method of encapsulating biologically active compounds on polymer carriers [6].

Pectin lowers the level of cholesterol in the blood, and this medicinal action is important in reducing the risk of cardiovascular disease. Some *in vivo* studies have determined that pectin is involved in cholesterol metabolism [7] [8]. When HM pectin was used, cholesterol levels in blood plasma and liver were observed to be more effectively lowered than when LM pectin was used [9].

Pectin-based hydrocolloid wound dressings are commercially available due to the properties of pectin, which enable this compound to be suitable for the production of hydrophilic wound dressings for absorbing inflammatory fluid, forming a barrier against bacteria, increasing the levels of growth factors, and binding medicinal active substances to the wound [10].

Pectin is degraded by the action of the microbiota in the large intestine; some parts of the side chain are de-esterified in the stomach at pH 2 - 4, and the β groups of the main chain are de-esterified in the small intestine at pH 4 - 6. Pectin is a water-soluble substance, and the pectin matrix acts as a carrier for the selective delivery of drugs to the colon and the gastrointestinal tract. In recent years, the nonabsorbable properties of pectin in the upper digestive tract have

been leveraged to develop drug-delivery mechanisms that selectively target the large intestine. The researchers found that the pectin matrix could be used in the form of tablets, drug carriers, and suspensions [11].

Pectin is a prebiotic carbohydrate. In addition, it provides health benefits such as accelerating the growth of beneficial bacteria, promoting the absorption of minerals, improving immunity, and reducing the risk of colorectal cancer. Pectin is not found in the upper digestive system. Based on these properties, pectin is used as a carrier for transporting medicinal substances [12]-[18]. One of the multifaceted challenges that limit the treatment of antibiotic resistance is the development of active antibiotic transport methods that enable drugs to reach (pathogenic) bacteria without harming healthy cells [19] [20] [21] [22] [23].

Therefore, using an active transport mechanism, pectin, which was used as a nanoparticle carrier to transport antibiotics, was extracted and encapsulated with antibiotics. The research was conducted to determine bacterial sensitivity to antibiotics.

2. Materials and Methods

This study was conducted using an experimental research design. The sea buckthorn was purchased from Mongolia and we sent the sea buckthorn to the laboratory, separated the sea buckthorn waste, and extracted the pectin.

We conducted the research in the Food Chemistry Laboratory of the Institute of Chemistry and Chemical Technology of MAS. This laboratory meets the "General requirements for testing and calibration of laboratory capabilities MNS ISO/IEC 17025: 2018". It was also carried out with the support of Etugen University Microbiology Laboratory, MNUMS Microbiology Laboratory.

2.1. Extracted Sea Buckthorn Waste

For separating waste from sea buckthorn, we follow the "Medical raw materials Dry fruit of *Hippophae rhamnoides* MNS 5225:2002" standard. Sea buckthorn was dried for 24 hours at −45˚C using a TFD5503 deep freezer dryer.

Separation of pectin by the ammonium oxalate method

To extract pectin from sea buckthorn waste, we followed the "method for determination of pectins MNS3080:1981" standard (**Figure 1**).

2.2. Preparation of a Solid Residue Insoluble in Alcohol

Before polysaccharide isolation, sea buckthorn solids were extracted with 95% ethanol and diluted with hydrochloric acid. For the preparation of acidified alcohol extracts, 50 g of the extract was acidified with 2 ml of concentrated HCl in 1 L of 95 percent (v/v) ethanol, and the solution was heated on a magnetic stirrer. The extracts from these two preparations were filtered, and the solid residue was dried in a petri dish.

Separation of polysaccharides

Thirty-five grams and 17.5 g of each of the dried residues of extracts I and II

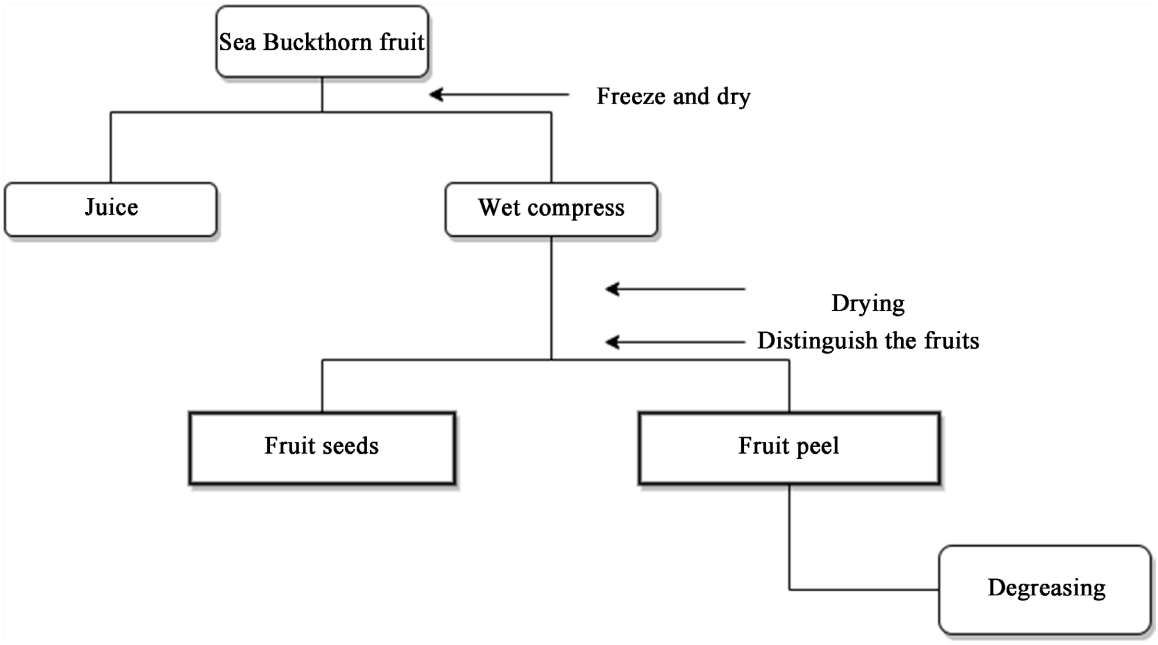

Figure 1. Schematic of the sample prepared passage.

were dissolved in 1 liter of 0.8% ammonium oxalate salt aqueous solution at 75˚C for 1 hour on a magnetic stirrer. As soon as extracts I and II had been separated, the extract from the residue was dissolved in 600 ml of 0.8% ammonium oxalate aqueous solution at 75˚C for 1 hour on a magnetic stirrer. Extracts I and II were filtered and precipitated with 1.2 liters of alcohol in a refrigerator at 4˚C for 72 hours. The sediment was filtered through filter paper, and pectin was separated.

2.3. Determination of the Chemical Properties of Pectin

Ash determination formula

Ash was calculated using the following formula.

$$X = a \cdot b \cdot 100 \quad (1)$$

Abbreviation: X—total mineral content (%)

a—ash weight (g)

b—sample weight (g)

Bertrand's method for the determination of simple carbohydrates

Multiplication of the volume of manganic acid (ml) used during the titration by 6.36 (the amount of copper in mg per ml of 0.1 n $KMnO_4$) was used to determine the amount of copper. The amount of copper in the titration sample was determined by using a special table that shows how much copper and how much glucose had been reduced.

Calcium pectate method for pectin quantification

The amount of pure pectin was multiplied by the coefficient of conversion to pectin acid, which is 0.9235. Afterward, the dilution factor and amount of pectin were carefully calculated.

Calculation of the levels of the free carboxyl group

The level of the free carboxyl group was calculated using the following formula.

$$K_{ch}\% = \frac{a}{p} \times 0.45 \quad (2)$$

Abbreviation: *a*—volume used for titration of 0.1 n NaOH (ml)

p—the amount of pectin analyzed (g)

Calculating the levels of the methylated carboxyl group

The levels of the methylated carboxyl group were calculated using the following formula.

$$K_M\% = \frac{b}{p} \times 0.45 \quad (3)$$

Abbreviation: *b*—the amount used during the 2nd titration of 0.1 n NaOH,

p—the amount of pectin initially analyzed in ml (g)

If the food pectin contains an acetyl group, the methylated carboxyl group is inserted into the gap, and the levels were determined by the following formula.

$$K_M = K_{ch} - K_c \quad (4)$$

Abbreviation: K_{ch}—free carboxyl group %

K_c—acetyl group %

Determination of total carboxyl group levels

The total level of the carboxyl group was calculated using the following formula.

The total level of the carboxyl group is equal to the sum of the levels of the free and methylated carboxyl groups.

Determination of the degree of esterification of pectin substances

The ISO standard for pectin is ISO 7623:2016, titled "Pectins - Determination of esterification degree." This standard provides a method for determining the degree of esterification of pectin, which is a measure of the degree to which pectin molecules are esterified with methoxyl groups.

The degree of esterification is defined as the ratio of methylated carboxyl groups to total carboxyl groups and is denoted by the letter λ.

$$\lambda = K_m K_n \times 100\% \quad (5)$$

Abbreviation: K_m—Methylated carboxyl group

K_n—Total carboxyl group

2.4. Methodology for Extracting Coacervate as a Drug Carrier

Preparation of microcapsules

When pectin-based microspheres were prepared, a polymer: antibiotic ratio of 1:1 was used. Chitazone (0.25 g) and pectin (0.25 g) were dissolved in 10 ml of water on a magnetic stirrer, 0.1 g and 0.25 g of antibiotics were added, and microcapsules were prepared by coacervate and water-based emulsion preparation methods [24].

Determination of physical and chemical properties of microcapsules

The physical and chemical properties of the extracted microcapsules were de-

termined by SEM.

2.5. Antibiotic Susceptibility Testing

The microdilution method was used to determine the antibiotic susceptibility of the isolates on Muller Hinton agar (Difco, Franklin Lakes, NJ, USA). Each isolated was tested for antibiotic susceptibility using a panel of the following antibiotics: pectin-based microsphere doxycycline hyclate, pure doxycycline hyclate (Biolab, Budapest, Hungary), and wontaxime (Daewon Pharm, Seoul, Korea). The microplates were incubated at 37˚C for 24 hours, and the inhibitory was measured. Interpretation of the results followed the criteria recommended by the Clinical Laboratory Standard Institute (MT100-S27). The antibacterial antibiotic was tested and validated on the standard strain of *Staphylococcus aureus* ATCC 29213 and MRSA ATCC 2758.

Preparation of pectin solution

The working solution was prepared by mixing 0.76 grams of powdered pectin with 1000 ml of distilled water.

Dilution

One hundred microlitres of Muller Hinton broth were placed in each of the first 4 rows of 96-well microplates, 100 µl of pectin solution was added as a negative control, and 10 µl of bacterial suspension prepared from each standard strain of *Staphylococcus aureus* ATCC 29213 and MRSA ATCC 2758 were diluted by 10 and stored at 37˚C were incubated in an incubator for 24 hours.

The amount of antibiotic we put in the pectin is shown in the dilution as 10^1 - 10^{10}. We prepared the working solution by mixing 0.76 grams of powdered pectin with 1000 ml of distilled water. A dilution was prepared from this sample. Negative control was performed by dissolving 1 gram of doxycycline hyclate and wontaxime powder in 1000 ml of distilled water and diluting it (Table 1).

Table 1. Antibiotic dilutions.

Dilution	Pectin /µg/	0.1 g doxy. containing microsphere/µg/	Doxycycline hyclate /µg/	Wontaxime /µg/
10^1	7.60	4.80	10.0	10.00
10^2	3.80	2.4	5.0	5.00
10^3	1.90	1.2	2.5	2.50
10^4	0.95	0.6	1.25	1.25
10^5	0.48	0.3	0.63	0.63
10^6	0.24	0.15	0.31	0.31
10^7	0.12	0.075	0.16	0.16
10^8	0.06	0.0375	0.08	0.08
10^9	0.030	0.0187	0.04	0.04
10^{10}	0.015	0.0093	0.02	0.02

2.6. Measurement of Bacterial Growth

Muller Hinton agar medium was prepared, and the medium was divided into 12 equal parts: negative control, positive control, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and incubated in an incubator for 24 hours at 37˚C. After culturing in dilutions, bacteria were evaluated by inoculating in a solid medium.

2.7. Statistical Analysis

Statistical analysis of the results in this research work was calculated using SPSS 25 in terms of both quantitative and qualitative variables. Qualitative variables were tested using ANOVA for frequency distribution, and quantitative variables were tested for location, centrality, and variance indicators using T-tests. A value of $p < 0.05$ was considered statistically significant.

3. Results

In this study, we extracted pectin from waste sea buckthorn peel by the ammonium oxalate method. We report its physical and chemical properties and parameters in Table 2.

Pectin is a brown, odorless, sour-tasting, dry powder. The degree of esterified pectin was 71.4%, the yield was 8.9%, the degree of free carboxyl groups was 1.3%, the degree of methylated carboxyl groups was 3.2%, the degree of total carboxyl groups was 4.5%, the degree of ash was 3.5% and the degree of nitrogen was 0.1% (Table 2).

During the study, the composition of pectin extracted from sea buckthorn peel was determined by electron microscopy, and it was found that 46.9% calcium was

Table 2. Physical and chemical parameters of pectin.

Physical parameters of pectin		
№	Parameter	Characteristics
1	Appearance	Brown homogeneous dry powder
2	Smell	Odourless
3	Taste	Sour taste
Chemical parameters of pectin		
№	Parameter	Contents
1	Exit	8.9
2	Free carboxyl group	1.3
3	A methylated carboxyl group	3.2
4	Total carboxyl group	4.5
5	Etherization	71.4
6	Ashes	3.5
7	Nitrogen	0.1

present in microspheres without doxycycline hyclate, 31.7% calcium was present in microspheres containing 0.1 g doxycycline, 53.9% of calcium was present in microspheres containing 0.1 g doxycycline hyclate (Table 3).

Table 4 shows the results of the determination of the sensitivity of bacteria to pectin-encapsulated doxycycline hyclate by the dilution method.

Pectin containing 0.1 g of doxycycline hyclate was evaluated. Bacterial growth inhibition was 0.8 times lower than with pure pectin. It was 1 time less than that of doxycycline hyclate alone, and 33 times lower than that of wontaxime. In a study of pectin containing 0.1 g of doxycycline hyclate against MRSA, bacterial growth was 6 times greater than that of pure pectin. This was followed by 2 times that of doxycycline hyclate alone, and 8 times that of wontaxime.

S. aureus growth was inhibited by pectin at a dose of 0.06 μg or 8 times that of MRSA at a dose of 0.08 μg with doxycycline hyclate. Pectin containing 0.1 g of doxycycline hyclate inhibited *S. aureus* and MRSA growth at different doses. In contrast, wontaxime is effective against *S. aureus* at 2.5 μg, which is 4 times higher than the MRSA dose.

Our sea buckthorn pectin shows activity against MRSA and *S. aureus*. But pectin containing 0.1 g of doxycycline hyclate showed the best results against MRSA and *S. aureus* at lower doses.

4. Discussion

As determined by the Mongolian standard MNS3078-1981, sea buckthorn peel

Table 3. Chemical composition of pectin-free and doxycycline-containing microspheres.

Chemical element	Pectin	DOXY-free microspheres	Microsphere containing 0.1 g of DOXY
Sodium	1.8	12.7	-
Magnesium	4.7	5	-
Silicone	2.3	3.3	4.8
Phosphorus	10.9	10.6	4.3
Sulfur	23.9	8.3	5.6
Chlorine	9.5	15.9	12.4
Calcium	46.9	31.7	53.9
Cali	-	12.5	17.7
Aluminium	-	-	1.3

Table 4. Determination of antibacterial activity.

№	Bacteria	Pectin	Pectin containing 0.1 g doxycycline hyclate	Doxycycline Hyclate	Wontaxime
1	*S. aureus*	0.06	0.075	0.08	2.5
2	MRSA	0.48	0.075	0.16	0.63

pectin is odorless, has a sour taste and no color, and is found in food substances. The total yield of pectin was 8.9 percent, which is close to the research value obtained by Banzragch D. *et al.*

Our research has the advantage that, compared with previous research (Banzragch D. *et al.*), the results take less time to access. The ideal conditions for pectin extraction vary from fruit to fruit, depending on their characteristics and the amount of pectin they contain.

El-Nawawi and Shehata obtained the highest yield of pectin from orange peel by extracting it at 90˚C for 120 minutes at pH 1.7 [25].

Pagan and Ibarz found that extracting pectin from peaches with 70% nitrogen at 80˚C and pH 1.2 for 60 minutes was more convenient than using citric acid instead of hydrochloric acid [26]. Additionally, S. Sangheetha and D.C.K. Illeperuma, who aimed to establish a suitable procedure for extracting pectin from mango fruit peel, the highest yield was 16.3% when extracted at pH 2.5 and 90˚C for 135 minutes, and the lowest yield of 13.1% was obtained at pH 3.7 and 60˚C when extracted for 90 minutes [27]. According to the results of these studies, the extraction time of pectin does not need to exceed 2 hours. The degree of esterification of soluble pectin is 71.4 percent, which shows that it can be used in food production.

According to Banzragch.D to *et al.*, the pectin yield was 7.38%, the free carboxyl group content was 3.95%, the esterification degree was 51.9%, the ash content was 5.4%, the nitrogen content was 0.1%. 3.2 percent, the total carboxyl group content was 4.5 percent, the degree of esterification was 71.4%, the degree of ash was 3.5 percent, and the degree of nitrogen was 0.1 percent [28].

Ma. Cristina B. Gragasin and Aileen R. Ligisan compared the color of apple pectin and mango pectin and observed that mango pectin is darker in color than apple pectin. The color of pectin dry powder isolated from sea buckthorn peels was different from that observed in other research results. Differences in research results may be due to differences between fruit types and species, the use of cultivated and wild fruits, and natural factors. Sea buckthorn produces orange pulp, while apples produce white pulp, mangos produce yellow pulp, and the color of pectin varies depending on the type of fruit [29].

In the study by Bernardo Bayóna and Verónica Bucalábc, phosphate and silver hybrid microspheres containing levofloxacin showed antibacterial activity several times stronger than that of levofloxacin alone. This was evaluated to determine the susceptibility of *Escherichia coli* and *Staphylococcus aureus* strains to antibiotics [30].

In the study by Demet Sensoy and Erdal Cevher, microspheres containing vancomycin hydrochloride in ratios of 1:1, 2:1, 3:1, and 4:1 were implanted in laboratory mice with artificial osteomyelitis caused by MRSA strains for 21 days. Compared with intramuscular injection of microspheres, implanted microspheres were more effective.

In the study by Demet Sensoya and Erdal Cevhera, artificial keratitis was established in the eyes of male rabbits with *Pseudomonas aeruginosa* and *Staphylococcus aureus*, and microspheres prepared in a 2:1 ratio of sulfanyl sodium

and polymer were suspended in oil and applied to the eyes for 2 days. On the 3rd and 6th days of antimicrobial treatment, clinical signs of keratitis, such as conjunctivitis or inflammation of the conjunctiva, ocular edema, and corneal infiltration, were diagnosed. These signs were detected when microspheres containing sulfanyl sodium dissolved in oil were used. By microbiological analysis, it was confirmed that the use of microspheres containing sulfanyl sodium in treatment is more effective than the use of sulfacyl sodium alone. This was confirmed when corneal pathogens were isolated and identified [31].

Microspheres made of ethyl cellulose have the advantages of stable drug transport, reduced frequency of daily intake, and lower treatment costs. Common pathogens were cultured at 37˚C for 24 hours, and inhibitory zone formation was measured using the positive control *Bacillus subtilis* strain at 22 ± 1.00, polysaccharide at 13 ± 1.14, and EC-THC using microspheres and collagen without inhibitory zone formation, EC-THC using polysaccharide and collagen hybrid microspheres. In the inhibitory zone, the level of formation was 13 ± 1.22. The *Pseudomonas aeruginosa* positive control showed 21 ± 1.13 and 12 ± 1.21 inhibitory zone formation on polysaccharide and collagen hybrid microspheres containing EC-THC, respectively, and no inhibitory zone formation on EC-THC-containing microspheres and collagen [32].

5. Conclusion

Pectin has a maximum yield of 1.3 during a 60-minute separation at pH 2 at a precipitation concentration ratio of 1:1.5. In the study, the precipitation concentration ratio of 1:1.5 and pH 2 were the most favorable conditions for extraction for 30, 60, and 120 minutes. Pectin alone showed antibacterial activity, and when the activity of pectin containing 0.1 g of doxycycline against *S. aureus* was determined, the inhibition of bacterial growth was 0.8 times lower than that achieved using pure pectin, 1 time lower than that of doxycycline alone, and 33 times lower than that of wontaxime. However, when the activity against MRSA was determined, bacterial growth was inhibited by 6 times less than pure pectin, 2 times less than doxycycline hyclate alone, and 8 times less than wontaxime.

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Authors' Contributions

Enkhtaivan Erdene conceived and designed this study; contributed to the acqui-

sition, analysis, and interpretation of the data; and was responsible for drafting, editing, and submission of the manuscript. Baatarkhuu Oidov and Enkhjargal Dorjbal had a significant influence on the study design and critical appraisal of the manuscript. Odonchimeg Munkhjargal contributed to the study design and acquired the samples. Nyamkhuu Lkhagva contributed to the interpretation of the data. Ariunsanaa Byambaa contributed to the study design and analysis and interpretation of the data and reviewed the manuscript. All of the authors reviewed, discussed, and approved the final manuscript.

Data Availability

All data generated or analyzed during this study are included in this published article.

Ethics Approval and Consent to Participate

The IRB Committee of the Mongolian National University of Medical Sciences approved the study protocol (№2021/3-02, approved on 06/04/2022).

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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