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RESEARCH ARTICLE

Competency of Lyophilization and Spray Drying Techniques to Improve the Solubility of Bosentan Monohydrate: A Comparative Study

Safvan Ali CHEMBAN[*], Lakhvir KAUR[**o], Gurjeet SINGH[***], Ravi Kumar DHAWAN[****] Anureet KAUR[*****] and Lovepreet SINGH[******]

Competency of Lyophilization and Spray Drying Techniques to Improve the Solubility of Bosentan Monohydrate: A Comparative Study

SUMMARY

The present study focused on comparing the efficacy of two novel techniques, lyophilization and spray drying, which were proposed to overcome the solubility drawbacks of the highly effective antihypertensive drug, bosentan monohydrate. Solid dispersion approach is the most globally acknowledged and successful method for improving solubility. Poloxamer 188 was used as the carrier to prepare the solid dispersions. The results indicated that the particle size, solubility, and dissolution profiles of formulated amorphous systems varied significantly. Lyophilized solid dispersions demonstrated the highest level of solubility in the prepared solid dispersions. The solid dispersion formulations FL10 and FS10 prepared using lyophilization and spray drying techniques were optimized using a 32 full factorial design approach. The resulting amorphous solid dispersions were characterized using Fourier-transform infrared spectroscopy (FTIR), particle size analysis, differential scanning calorimetry (DSC), X-ray diffraction (XRD), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). The optimized solid dispersion (FL10) prepared via lyophilization had an average particle size of 450.9 nm in particle size analysis. X-ray diffraction analyses of both FL10 and FS10 revealed a decrease in peak intensity compared to the drug and polymer, indicating the transformation of the crystalline form to amorphous. The outcomes of this study allow us to conclude that even though lyophilization and spray drying can be used to enhance solubility, lyophilization showed superior results.

Key Words: *Solid dispersion, lyophilization, spray drying, solubility enhancement, hypertension*

Bosentan Monohidratın Çözünürlüğünü Artırmak için Liyofilizasyon ve Püskürtmeli Kurutma Tekniklerinin Yeterliliği: Karşılaştırmalı Bir Çalışma

ÖZ

Bu çalışma, yüksek etkili antihipertansif bir ilaç olan bosentan monohidratın çözünürlük sorunlarının üstesinden gelebilmek için 2 yeni teknik olan liyofilizasyon ve püskürterek kurutma tekniklerinin etkileri üzerine odaklanmıştır. Katı dispersiyon yaklaşımı, çözünürlük arttırmak için dünya çapında en çok kabul gören başarılı bir yöntemdir. Poloxamer 188, katı dispersiyonları hazırlamak için taşıyıcı olarak kullanılmıştır. Sonuçlar göstermektedir ki formüle edilmiş amorf sistemlerin partikül boyutu, çözünürlüğü ve çözünme profilleri önemli ölçüde değişmiştir. Liyofilize katı dispersiyonlar, hazırlanan katı dispersiyonlarda en yüksek çözünürlük seviyesini göstermiştir. Liyofilizasyon ve püskürterek kurutma teknikleri kullanılarak hazırlanan katı dispersiyon formülasyonları FL10 ve FS10, 32 tam faktöriyel tasarım yaklaşımı kullanılarak optimize edilmiştir. Hazırlanan amorf katı dispersiyonlar Fourier-transform kızılötesi spektroskopisi (FTIR), partikül büyüklüğü analizi, diferansiyel taramalı kalorimetri (DSC), X-ışını difraksiyonu (XRD), taramalı elektron mikroskobu (SEM) ve transmisyon elektron mikroskobu (TEM) kullanılarak karakterize edilmiştir. Liyofilize edilmiş optimum katı dispersiyonların (FL10) ortalama partikül büyüklüğü 450.9 nm'dir. Hem FL10 hem de FS10'un X-ışını kırınım analizleri, ilaca ve polimere kıyasla tepe yoğunluğunda bir azalma ortaya çıkarmıştır ve bu, kristalli formun amorfa dönüşümünü göstermetedir. Bu çalışmanın sonuçları, çözünürlüğü arttırmak için liyofilizasyon ve püskürterek kurutma kullanılabilse de liyofilizasyonun daha iyi sonuçlar gösterdiği sonucuna varmamızı sağlar.

Anahtar Kelimeler: *Katı dispersiyon, liyofilizasyon, püskürterek kurutma, çözünürlüğü artırma, hipertansiyon*

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INTRODUCTION

Hypertension refers to persistently increased blood pressure (BP) in the systemic arteries. According to the Global Burden of Disease study, non-optimal blood pressure continues to be the single most significant risk factor contributing to the global burden of disease and all-cause mortality, accounting for 9.4 million deaths and 212 million lost healthy life years (8.5% of the global total) each year (Forouzanfar, 2015). Worldwide, hypertension is the most prevalent preventable risk factor for cardiovascular disease (CVD), chronic kidney disease, and cognitive impairment, and is the single leading cause of death and disability of all reasons (Forouzanfar, 2015).

One type of hypertension that affects the lungs and the heart is pulmonary arterial hypertension (PAH), which falls under the pulmonary hypertension classification. PAH is defined as a persistent elevation of pulmonary arterial pressure to greater than 25 mm Hg at rest or greater than 30 mm Hg during exercise, with a mean pulmonary capillary wedge pressure and a left ventricular end-diastolic pressure less than 15 mm Hg (Gaine, 1998). This differ from having normal blood pressure. When the small arteries in the lungs become narrowed or obstructed in a patient with PAH, blood has a more challenging time flowing through them, increasing the blood pressure in the lungs (Farber, 2004). PAH is a well-established multifactorial clinical condition that is severe and occasionally fatal. Because endothelial dysfunction, vasoconstriction, inflammatory responses, and platelet aggregation are the primary pathophysiological arms of PAH, specific therapeutic techniques have been developed to suppress these disorders. These therapies are highly effective at treating the disease.

Bosentan Monohydrate (BM) is a dual endothelin receptor antagonist that can be effectively used to treat PAH by inhibiting the action of endothelin molecules that cause blood vessel narrowing and hypertension in the absence of endothelin molecules. It improves patients' exercise capacity and slows the rate of clinical deterioration. Patients with PAH have elevated endothelin levels in their plasma and lung tissue, which is a potent vasoconstrictor (McLaughlin, 2006). BM prevents endothelin from binding to its receptors, neutralizing its detrimental effects. It is a water-insoluble compound having 50% absolute bioavailability (Dingemanse, 2004). It possess several limitations like insufficient absorption, fluctuating bioavailability, and gastrointestinal toxicity due to its water insolubility. As a result, increasing drug solubility is vital for achieving the desired drug concentration in the systemic circulation.

Solid dispersion is an effective technique to enhance solubility and thus increases the bioavailability of drugs. Reduced particle size, improved wettability, and increased porosity are just a few of the advantages of solid dispersion (Singh, 2011). Solid dispersions can easily be formulated into tablets or capsules for oral administration and ensure the stability of the drugs even in amorphous form (Chiou, 1971). Solid dispersions can be prepared by numerous techniques like solvent evaporation method, fusion method, melting method, kneading method, co-grinding method, melt agglomeration and hot-melt extrusion, etc. All the methods have been reported to enhance the solubility of poorly water-soluble drugs. Still, an attempt has been made in the present study to compare the efficacy of lyophilization and spray drying techniques.

MATERIALS AND METHODS

Bosentan monohydrate was purchased from Pure Chem Pvt. Ltd., Gujarat. Poloxamer 188 was purchased from Alfa Aesar, Massachusetts, United States. Methanol was obtained from Qualikems Fine Chem. Pvt. Ltd., Gujarat. Chloroform was purchased from Merck Specialities Pvt. Ltd., New Delhi. All other chemicals used were of analytical grade.

Solubility study

The solubility of BM in various solvents was evaluated using the shake flask method. An excess of the drug was added to screw-capped vials containing 10 ml of each solvent and then kept in a water bath shaker at 37±1°C for 48 h. Then the saturated solutions

were filtered through 0.45 µm sized membrane filters and analyzed using a UV spectrophotometer (UV-1800, Shimadzu Corporation, Kyoto, Japan) at 268 nm and resolution 0.2 nm (Patel, 2008). The method was validated for linearity, accuracy, and precision. The linearity range was found to be 5-40 µg/ml, with a % RSD(Relative standard deviation) value less than two, which indicates the method is precise. The method's sensitivity was found by determining LOD (lower limit of detection) and LOQ (lowest limit of quantification). The LOD and LOQ values were 0.95 and 2.88 µg/ml, respectively.

Drug-excipient compatibility studies

While designing the solid dispersions, it is imperative to consider the compatibility of drugs and polymers used within the systems. It is therefore, necessary to confirm that the drug does not show any incompatibility with the polymer under experimental conditions (40±5°C and 75±5% RH) for at least three weeks. The desired quantity of drug with specified excipient poloxamer 188 (P188) was taken in the ratio of 1:5 and mixed thoroughly, sieved, and filled in dried vials. The vials were examined daily at regular intervals for discoloration, clump formation, and liquefaction. Also, the FTIR spectra of pure drug, polymer, and solid dispersion were obtained to determine the compatibility. For FTIR, a sample of approximately 4 mg was kept in an FTIR spectrometer, and the spectra were recorded (Maximiano, 2011).

Preparation of solid dispersion

Lyophilization (freeze-drying method)

Solid dispersions were prepared by the freeze-drying method. BM was dissolved in a sufficient quantity of methanol to form phase I. Similarly, (P188) were dissolved separately in water to form phase II, and both phases were mixed. Methanol was evaporated and the resulted solution was frozen in a quick/deep freezer at −20°C and was then lyophilized in a freeze dryer (LyoQuest-55 Azbil Telstar Technologies, Terrassa, Spain) at temperatures of −30°C to −40°C and a vacuum of 0.200 mbar. The freeze-dried mass was then sieved through sieve no. 86 and stored in a desiccator (Betageri, 1995; Abdul-Fattah, 2002).

Spray drying method

Solid dispersions were also prepared by the spray drying method. BM was dissolved in a sufficient quantity of methanol to form phase I. Similarly, P188 was dissolved separately in water to form phase II, and both phases were mixed and sonicated for 2 minutes. The resultant solutions are then spray-dried using a spray dryer (Spray Mate Lab Spray Dryer, JISL, Mumbai, India) at inlet temperatures of 90°C to 110°C with an outlet temperature of 80°C at a feed rate of 10 ml/min and aspiration speed of 35 mbar. The spray-dried mass was then sieved through sieve no. 86 and stored in a desiccator (Paradkar, 2004; Ha, 2014).

Characterization of solid dispersion formulation

The drug content of all the prepared solid dispersions was determined by dissolving solid dispersions equivalent to 10 mg of BM according to their ratio prepared in methanol. It was then diluted to obtain a theoretical concentration of 10 µg/ml. The solution was then filtered through membrane filters and analyzed with a UV spectrophotometer. Then the percentage yield of each formulation was determined according to the final weight of solid dispersions.

$$Percentage\ yield = \frac{Practical\ weight\ of\ solid\ dispersion}{Theoretical\ weight\ of\ solid\ dispersion} \times 100$$

Solubility of prepared solid dispersion formulations

The solubility of solid dispersion in distilled water was evaluated. Excess of the solid dispersions was added to screw-capped vials containing 10 ml of distilled water and then kept on a water bath shaker at 37±1°C for 48 h. Then the saturated solutions were filtered through 0.45 µm sized membrane filters and analyzed using a UV spectrophotometer.

***In vitro* drug release study**

In vitro drug release of prepared solid dispersions and the pure drug was performed in triplicate using a dissolution apparatus (DS 8000, Labindia Analytical

Instruments Pvt. Ltd., Navi Mumbai, India) in PBS pH 6.8 at 37±0.5°C using USP type II apparatus at 100 rpm. Powdered solid dispersions equivalent to 62.5 mg of BM were added to the dissolution medium. At appropriate time intervals, 5 ml of the sample was withdrawn and replaced with a fresh dissolution medium to maintain the sink conditions. The withdrawn samples were filtered using a membrane filter and analyzed for drug content using a UV spectrophotometer. The dissolution efficiency (DE%) after 120 min was determined via the trapezoidal method and was calculated as the percentage area of a rectangle divided by the area of 100% dissolution at a particular time (Potluri, 2011; Krupa, 2017).

Experimental design of solid dispersions

Optimization of the lyophilization process and spray drying process

A 3^2 full factorial design (Design-Expert version 11; State Ease Inc., USA) was used to determine the optimized formulation to target percentage yield and highest percentage dissolution efficiency in the case of lyophilized and spray-dried solid dispersions. In this design, two factors were evaluated, each at three levels and experimental trials were performed at all nine possible combinations. For the lyophilization process, the drug to polymer ratio (X_1) and temperature (X_2) was considered as independent variables, whereas, DE_{120}% and Yield% were taken as dependent variables. In the case of the spray drying process, the drug to polymer ratio (X_1) and inlet temperature (X_2) were considered as independent variables, whereas, the DE_{120}% and Yield% were taken as dependent variables. In both cases, a checkpoint batch was prepared to prove the validity of the evolved mathematical model. In addition, contour plots were used to graphically represent the effect of independent variables (Singh, 2017).

The desirability of all solid dispersions using optimization software

A numerical optimization technique utilizing the desirability functions approach was used to generate the optimum settings for the process conditions of both preparation methods of solid dispersions. All response variables were optimized using the desirability functions approach with the Design-expert software version 11. The solid dispersion having the maximum desirability value was considered as the optimal formulation.

Characterization of optimized formulations using different techniques

IR spectral analysis

The chemical interactions and compatibility of BM, P188, and optimized formulations were determined using FTIR analysis. The samples were mixed with 80 mg of dry potassium bromide (KBr), and the mixture was compressed into discs. Later, the discs were scanned in the wavelength of 4000-500 cm^{-1}.

Particle size and size distribution analysis

The particle size of the optimized solid dispersions was determined using a particle size analyzer (Malvern Zetasizer Nano ZS90, United Kindom). The samples were suspended in triple distilled water and subjected to particle size analysis. This method also depicted the polydispersity index, which is a measure of uniformity in size distribution.

Differential scanning calorimetry (DSC)

DSC analysis was performed using an automatic differential scanning calorimeter (DSC822e, Mettler Toledo, Ohio, United States). Each sample of 3 mg was weighed and analyzed in pierced aluminum pans at a heating rate of 10°C/min and temperature range of 10 to 300°C.

X-ray diffraction (XRD)

The crystalline nature of the drug can be confirmed using an X-ray diffractometer (D/max r-B, Rigaku, Japan). XRD analysis of drug, polymer, and solid dispersions was performed using Cu-Kα radiation at an of angle 2θ range from 5° to 80° with a step size of 0.02°, a step time of 18.7 min, and a scanning speed of 5°/min.

Scanning electron microscopy (SEM)

A scanning electron microscope (SUPRA-55; Zeiss, Germany) was used to examine the morphology of pure drug, polymer, and prepared solid dispersions at an accelerating voltage of 10 kV and an aperture of 20 μm. The sample powder was mounted on a brass stub with graphite glue and then slathered with gold under vacuum before being viewed under SEM.

Transmission electron microscopy (TEM)

The morphology (particle shape and size) of the optimized solid dispersion was determined using TEM. The solid dispersion was dispersed in triple distilled water. A drop was placed on a carbon-coated copper grid and dried before being examined under a transmission electron microscope (HRTEM, JEM 2100, JEOL Ltd., Tokyo, Japan), which was operated at a 200 kV accelerating voltage and a beam current of 100 μa (Ricarte, 2015).

Stability study of optimized solid dispersion

During the preparation of solid dispersions, the drug undergoes a transition from crystalline form to amorphous form. But during storage, they are widely reported to recrystallize. So, accelerated stability studies were conducted. The optimized solid dispersions were stored at room temperature for 3 months. The dispersions were analyzed for changes in physical appearance and drug content after a period of 0, 30, 45, 60, and 90 days. After a storage period of 3 months, X-ray diffraction and particle size studies were conducted to determine any changes in particle size and crystallinity.

Statistical analysis

The data is provided as the mean and standard deviation of three sets of results. Analysis of variance (ANOVA) was used to examine the statistical difference between solubility and dissolution efficiency, followed by Tukey's test (Sigma stat 3.5; STATCON). At the 0.05 level of probability, significance was determined. A 3^2 full-factorial design (Design-Expert version 11; State Ease Inc., USA) was used to investigate the influence of formulation variables on the optimization process to obtain the desired formulation.

RESULTS AND DISCUSSION

Solubility profiles

The solubility of the BM in a variety of solvents was determined, including distilled water, methanol, phosphate buffer 6.8, and phosphate buffer 1.2. The lowest solubility of the BM was found to be 10.19±0.3 μg/ml in distilled water, while the highest solubility was found to be 3090.7±15.6 μg/ml in methanol. The solubility of the BM in phosphate buffer 6.8 and phosphate buffer 1.2 was found to be 48.1±2.1 and 10.54±0.5, respectively. The results show that the drug is completely soluble in methanol, insoluble in water, and shows pH-dependent solubility as reported (Krupa, 2017).

Drug-excipient compatibility studies

In the drug-excipient compatibility study, the desired quantity of drug with excipients was kept under observation for three weeks for any physical changes. There were no physical changes for three weeks. As shown in Figure 1, FTIR confirmed that characteristic peaks of BM and P188 seem to be preserved in prepared solid dispersion, which proves that there was no chemical interaction between the drug and the excipient.

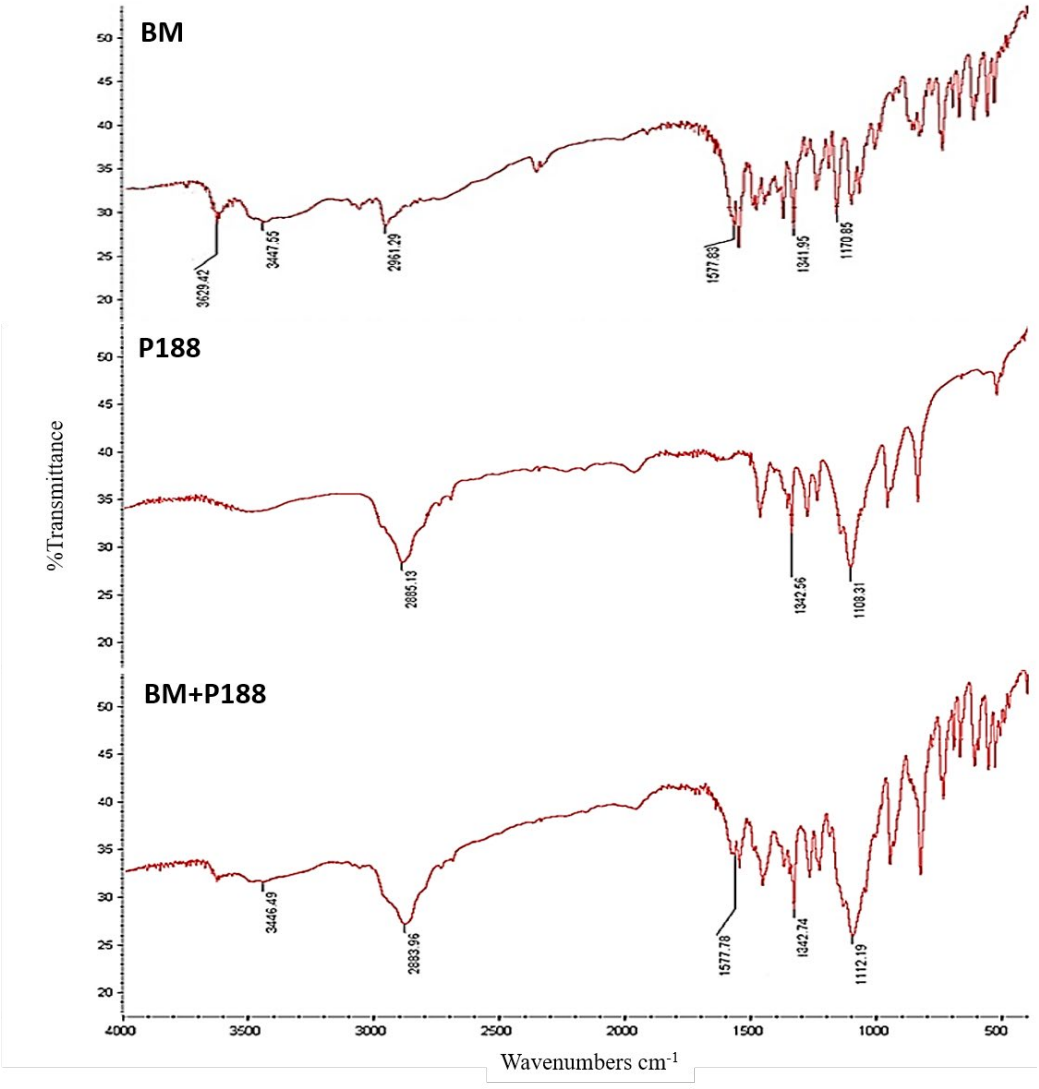


Figure 1. FTIR of bosentan monohydrate, poloxamer 188, and bosentan monohydrate-poloxamer 188

BM: Bosentan monohydrate, P188: Poloxamer 188

Preparation of solid dispersions

The solid dispersions were prepared by using lyophilization and spray drying methods. Lyophilized solid dispersions were prepared at the drug to polymer ratios of 1:1, 1:2, and 1:3 and at temperatures of -30°C, -35°C, and -40°C. Whereas, spray-dried solid dispersions were prepared with the drug to polymer ratios of 1:1, 1:2, and 1:3 at 90°C, 100°C, and 110°C inlet temperatures. Nine formulations using each technique were prepared where lyophilized solid dispersions were coded as FL1, FL2,...FL9 and similarly spray-dried solid dispersions were coded as FS1, FS2,...FS9. Compositions of all solid dispersions are shown in Table 3.

Then the percentage yield (Yield %) and percentage drug content (DC %) of each formulation were determined. As shown in Table 3, it was found that as the amount of polymer increased, the Yield % decreased. It might be due to the sticky nature of P188. Solid dispersion formulations prepared by

using lyophilization have shown a higher Yield % than the solid dispersion formulation prepared by the spray drying method. All formulations have shown an average DC % of 95% regardless of their Yield %.

The solubility of all solid dispersions prepared using lyophilization and spray drying was evaluated in distilled water. As depicted in Table 1, both methods demonstrated a significant increase in solubility as the amount of polymer increased. However, there was no significant increase in solubility after the drug to polymer ratio 1:2 in either method. When the preparation methods were compared, lyophilization outperformed spray drying at the same drug to polymer ratios. This may be because the lyophilization process produces a porous and fluffy product, increasing the surface area and thus the surface free energy, resulting in increased solubility (Betageri, 1995).

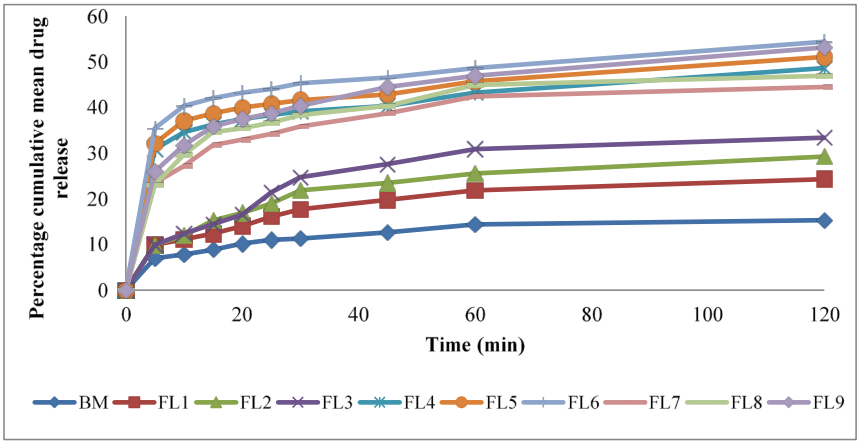
Table 1. Composition and characterization of prepared solid dispersions of bosentan monohydrate

Composition			Characterization			
Formulation code	**Drug: Polymer ratio**	**Temperature/ inlet temperature (℃)**	**%Yield**	**%DC**	**Saturation solubility (µg/ml)**	**%DE_{120}**
Bosentan monohydrate (Pure Drug)						
BM	-	-	-	-	10.19±0.3	
Lyophilization						
FL1	1:1	-30	93.7±1.8	98.1±1.9	213±1.9	19.5±1.03
FL2	1:1	-35	94.6±1.3	97.1±1.0	219±2.35	23.9±1.07
FL3	1:1	-40	93.1±0.8	99.0±1.5	222±1.26	26.62±0.80
FL3	1:2	-30	88.3±0.4	96.8±1.4	308±2.73	41.36±1.99
FL4	1:2	-35	87.1±1.1	99.0±1.4	310±4.28	43.72±1.55
FL5	1:2	-40	86.1±0.6	99.4±1.9	313±2.82	46.9±0.55
FL6	1:3	-30	79.2±1.1	97.3±1.6	326±4.16	38.43±0.46
FL8	1:3	-35	78.1±1.6	98.5±0.9	327±3.47	40.64±1.48
FL9	1:3	-40	77.6±0.9	97.9±1.1	329±2.62	43.94±1.97
Spray drying method						
FS1	1:1	90	74.2±0.8	97.2±1.3	195±2.76	21.85±0.39
FS2	1:1	100	73.3±1.1	94.9±1.8	201±1.22	23.8±0.64
FS3	1:1	110	74.9±0.6	95.7±1.9	205±1.76	25.86±1.68
FS3	1:2	90	61.3±1.5	98.5±1.1	296±4.16	38.63±1.46
FS4	1:2	100	63.4±0.9	98.2±1.4	302±2.89	40.61±1.18
FS5	1:2	110	62.2±1.0	97.1±1.5	308±3.01	41.75±0.94
FS6	1:3	90	60.5±0.2	96.3±1.2	322±3.98	38.2±1.12
FS8	1:3	100	59.3±1.8	96.1±1.1	326±3.56	38.71±1.58
FS9	1:3	110	58.4±0.9	95.4±1.1	328±3.15	39.53±0.89

Data are expressed as mean ±SD (n=3); Yield %: Percentage yield, DC %: percentage drug content, DE_{120}%: Dissolution efficiency after 120 min

***In vitro* dissolution studies**

In the case of BM, it showed a percentage cumulative drug release of 15.30%, whereas all solid dispersions showed an extended drug release rate over the period of 120 min. In the case of lyophilized solid dispersions, it showed percentage cumulative drug release ranging from 24.31% to 54.34%, whereas, in the case of spray-dried solid dispersions, they have shown the percentage cumulative drug release ranging from 25.13% to 44.88%, as shown in Figure 2. Over a period of 120 min, neither pure drug nor solid dispersions have shown a 100% cumulative drug release. In all solid dispersions, as the amount of polymer increased, the DE_{120}% also increased as shown in Table 3. When we compared the solid dispersions, the lyophilized solid dispersions have shown an increased DE_{120}% compared to the spray-dried solid dispersions at the same drug to polymer ratios. This increase in the dissolution rate can be attributed to an increase in solubility, which was found to be better in the case of spray-dried solid dispersions. To deep root these findings, the formulations were further optimized and tested for other parameters like particle size analysis, differential scanning calorimetry (DSC), X-ray diffraction (XRD), scanning electron microscopy (SEM), and transmission electron microscopy (TEM) (Dangre, 2017).



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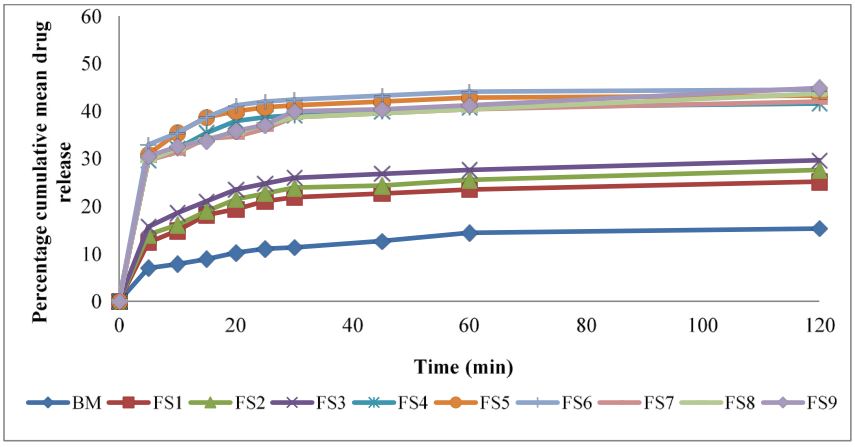

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Figure 2. Graphical representation of dissolution release profile of pure bosentan monohydrate and solid dispersions prepared by (A) lyophilization and (B) spray drying

Experimental design of solid dispersions

Optimization of the lyophilization method

A 3^2 full factorial design approach was used to determine the optimized formulation having maximum Yield % and maximum DE_{120}% by using design expert software (Design-Expert 11). Drug to polymer ratio (X_1) and temperature (X_2) were taken as two independent variables. A statistical model incorporating both interactive and polynomial terms was used to estimate the response by using the equation.

$$Y= b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_1^2X_1^2 + b_2^2X_2^2$$

Y is the dependent variable (Y_1 = Yield % and Y_2 = DE_{120}%), b_0 is the arithmetic mean response of all 9 runs, b_1 and b_2 are estimated coefficients for X_1 and X_2, respectively. Here X_1 and X_2 provide the average result on varying a single factor at one time, whereas X_1X_2 is the interaction term that illustrates how the response changes when 2 factors are changed simultaneously. Both polynomial terms i.e., $(X_1)^2$ and $(X_2)^2$ are included in determining nonlinearity.

The blueprint and results of lyophilized solid dispersions are shown in Table 2. There was a significant difference in Yield% (94.6±1.3% to 77.6±0.9%) and DE_{120}% (19.5±1.03% and 46.9±0.55%) in all the prepared formulations.

Table 2. Blueprint of 3^2 full factorial design (lyophilized solid dispersions)

Formulation code	Variable levels in coded form		Yield%	DE_{120}%
	X_1	X_2		
FL1	-1	-1	93.7±1.8	19.5±1.03
FL2	-1	0	94.6±1.3	23.9±1.07
FL3	-1	1	93.1±0.8	26.62±0.80
FL4	0	-1	88.3±0.4	41.36±1.99
FL5	0	0	87.1±1.1	43.72±1.55
FL6	0	1	86.1±0.6	46.9±0.55
FL7	1	-1	79.2±1.1	38.43±0.46
FL8	1	0	78.1±1.6	40.64±1.48
FL9	1	1	77.6±0.9	43.94±1.97
TCP (check point)	**0.19**	**1.0**	**90.8±0.9**	**43.2±0.61**
Coded values	**Actual values**			
	X_1 (Ratio)	**X_2 (Temp.)**		
-1	1:1	-30°C		
0	1:2	-35°C		
1	1:3	-40°C		

Data are expressed as mean ±SD (n=3); Yield%: Percentage yield, DE_{120}%: Dissolution efficiency after 120 min

Table 3. Regression analysis data of lyophilized solid dispersions

Response	Yield%		DE_{120}%	
	FM	RM	FM	RM
b_0	92.40	91.97	43.97	36.11
b_1	-4.25	-4.25	8.83	8.83
b_2	-0.23	-0.23	3.03	3.03
b_{11}	-0.25		-11.82	
b_{22}	-0.40		0.03	
b_{12}	0.25		-0.40	

Yield%: Percentage yield, DE_{120}%: Dissolution efficiency after 120 min, FM: Full model, RM: Reduced model

It is depicted from Table 2 that both the chosen independent variable have a significant effect on Yield% and DE_{120}%. The fitted equation (full and reduced model) relating different responses, Yield%, and DE_{120}% to the transforming factor is revealed in Table 3.

The polynomial equations can be utilized to draw conclusions from the magnitude of coefficient and positive or negative sign. The results of the ANOVA as depicted in Table 4 were executed to identify the insignificant factors. The value of correlation was near 1 for both Yield% and DE_{120}%, thereby indicating a

good fit for all the dependent variables. Among both dependent variables, regression analysis suggests that coefficients b_{11}, b_{22}, and b_{12} ($P \geq 0.05$) were insignificant in predicting Yield% and DE_{120}%. Hence these terms were omitted from the full model to generate the reduced model.

Both coefficients b_1 and b_2 bear a negative sign as shown in multiple linear regression analysis (reduced model), which indicates that upon increasing the drug to polymer ratio or temperature, Yield% decreases. On the contrary, the increase in drug to polymer ratios and the temperature increased the DE_{120}% as the coefficients b_1 and b_2 bear positive signs.

Table 4. Analysis of variance of the full model and the reduced model for the dependent variables in the case of lyophilized solid dispersions of bosentan monohydrate

Full model	For Yield%				
	df	**SS**	**MS**	***f***	**R^2**
Regression	5	109.40	21.88	33.43	0.9824
Residual	3	1.96	0.65		
Reduce model					
Regression	2	108.70	54.35	122.67	0.9761
Residual	6	2.66	0.44		
Full model	**For DE_{120}%**				
	df	**SS**	**MS**	***f***	**R^2**
Regression	5	803.17	160.63	492.87	0.9988
Residual	3	0.977	0.032		
Reduce model					
Regression	2	523.01	261.51	5.58	0.9504
Residual	6	281.13	46.86		

df: Degree of freedom, SS: Sum of squares, MS: Mean of squares, *f:* Fischer's ratio, R: Regression coefficient

Optimization of formulation variables of lyophilization method

The optimization of lyophilized solid dispersions' components (Drug to polymer ratio and temperature) was done to target the Yield% and DE_{120}% of 93% and 45%, respectively. The optimized amount determined with the help of software is depicted in surface response curves as shown in Figure 3. A checkpoint batch (TCP) was prepared at $X_1 = 0.19$ level and $X_2 = 1.0$ level at which Yield% and DE_{120}% were 90.8±0.9 and 43.2±0.61, respectively. The optimized batch (TCP) depicted the expected results. The desirability of the optimized batch was 0.914727.

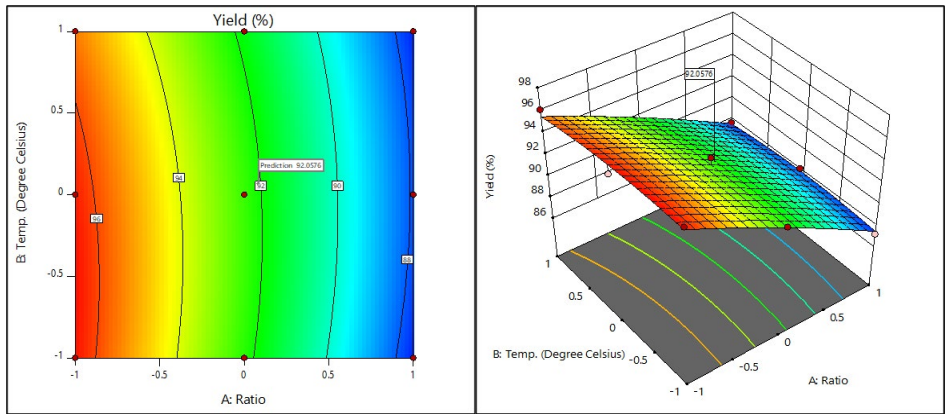
From the optimization conducted, it has displayed the optimized data in a coding form. The coded responses to X_1 and X_2 from the input data in the 3^2 full factorial design were 0.19 and 1.0, respectively. The responses were decoded and were found to be 1:2.2 and -40, i.e., for maximum %Yield and $\%DE_{120}$, the drug: polymer ratio and the temperature of the lyophilization method should be 1:2.2 and -40°C, respectively. To evaluate the prediction capability of the models and to verify the optimization process, lyophilized solid dispersions were prepared based on optimal process variable settings.

Optimization of the spray drying process

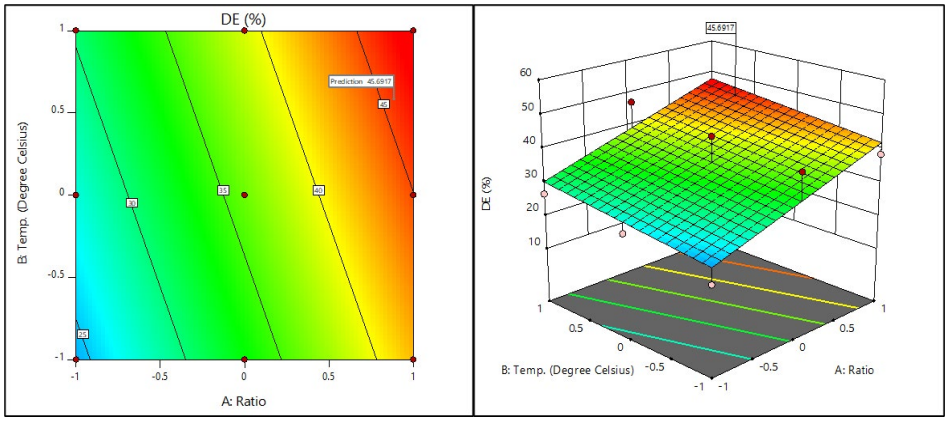
Using design expert software (Design Expert 11), a 3^2 full factorial design approach was used to find the optimal formulation with maximum Yield% and DE_{120}%. Two independent variables were used and they are drug: polymer ratio and inlet temperature. A statistical model incorporating both interactive and polynomial terms was used to estimate the response by using the equation.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_1^2X_1^2 + b_2^2X_2^2$$

Y is the dependent variable (Y_1 = Yield% and Y_2 = DE_{120}%), b_0 is the arithmetic mean response of all 9 runs, b_1 and b_2 are estimated coefficients for X_1 and X_2, respectively. Here X_1 and X_2 provide the average result on varying a single factor at one time, whereas X_1X_2 is the interaction term that illustrates how the response changes when 2 factors are changed simultaneously. To determine nonlinearity, both polynomial terms $(X_1)^2$ and $(X_2)^2$ are used.



A



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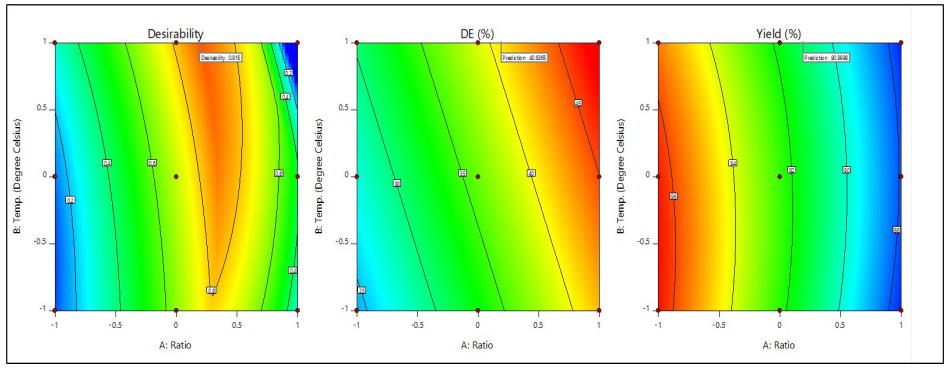

C

Figure 3. Response surface plots of (A) percentage yield, (B) percentage dissolution efficiency, and all response surface plots of (C) lyophilized solid dispersions using optimization software

The blueprint and results of spray-dried solid dispersions are shown in Table 5. There was a significant difference in Yield% (74.2±0.8% to 58.4±0.9%) and DE_{120}% (21.85±0.39% and 39.53±0.80%) in all the prepared formulations.

Table 5. Blueprint of 3^2 full factorial design (spray-dried solid dispersions)

Formulation code	Variable levels in coded form		Yield%	DE_{120}%
	X_1	X_2		
FS1	-1	-1	74.2±0.8	21.85±0.39
FS2	-1	0	73.3±1.1	23.8±0.64
FS3	-1	1	74.9±0.6	25.86±1.68
FS4	0	-1	61.3±1.5	38.63±1.46
FS5	0	0	63.4±0.9	40.61±1.18
FS6	0	1	62.2±1.0	41.75±0.94
FS7	1	-1	60.5±0.2	38.2±1.12
FS8	1	0	59.3±1.8	38.71±1.58
FS9	1	1	58.4±0.9	39.53±0.80
TCP (check point)	**0.29**	**0.99**	**68.4±0.7**	**40.2±0.5**
Coded values	**Actual values**			
	X_1 (Ratio)	**X_2 (Inlet Temp.)**		
-1	1:1	90°C		
0	1:2	100°C		
1	1:3	110°C		

Data are expressed as mean ±SD (n=3); Yield%: Percentage yield, DE_{120}%: Dissolution efficiency after 120 min.

It is portrayed clearly from Table 5 that both the chosen independent variables have a significant effect on Yield% and DE_{120}%. Table 6 shows the fitted equation (full and reduced model) relating various responses, Yield %, and DE_{120}% to the transforming factor.

Table 6. Regression analysis data of spray-dried solid dispersions

Response	Yield%		DE_{120}%	
	FM	**RM**	**FM**	**RM**
b_o	68.51	69.69	40.38	34.33
b_1	-5.70	-5.70	7.49	7.49
b_2	-0.46	-0.46	1.41	1.41
b_{11}	0.23		-9.00	
b_{22}	1.53		-0.07	
b_{12}	0.30		-0.67	

Yield%: Percentage yield, DE_{120}%: Dissolution efficiency after 120 min, FM: Full model, RM: Reduced model

The polynomial equations can derive conclusions based on the coefficient magnitude and positive or negative sign. ANOVA results, as shown in Table 7, were used to identify insignificant factors. The correlation value was close to one for both Yield% and DE_{120}%, indicating a good fit for all dependent variables. Regression analysis showed that coefficients b_{11}, b_{22}, and b_{12} ($P \geq 0.05$) were insignificant in predicting Yield% and DE_{120}% among both dependent variables. As a result, these terms were omitted from the full model to generate the reduced model.

Both coefficients b_1 and b_2 bear a negative sign as shown in multiple linear regression analysis (reduced model), which indicates that upon increasing the drug: polymer ratio or inlet temperature, Yield% decreases. On the contrary, the increase in drug to polymer ratios and inlet temperature increased the DE_{120}% as the coefficients b_1 and b_2 bear positive signs.

Table 7. ANOVA results of the full model and the reduced model for the dependent variables in the case of spray-dried solid dispersion of bosentan monohydrate

Full model	For Yield%				
	df	**SS**	**MS**	***f***	**R^2**
Regression	5	201.42	40.28	18.28	0.9882
Residual	3	6.61	2.20		
Reduce model					
Regression	2	196.25	98.12	49.97	0.9434
Residual	6	11.78	1.96		
Full model	For DE_{120}%				
	df	**SS**	**MS**	***f***	**R^2**
Regression	5	512.36	102.47	1590.10	0.9996
Residual	3	0.19	0.06		
Reduce model					
Regression	2	348.38	174.19	6.37	0.9797
Residual	6	164.18	27.36		

df: Degree of freedom, SS: Sum of squares, MS: Mean of squares, *f*: Fischer's ratio, R: Regression coefficient

Optimization of formulation variables of spray drying method

The optimization of spray-dried solid dispersions components (drug to polymer ratio and inlet temperature) was done to target the Yield% and DE_{120}% of 70% and 41%, respectively. The optimized amount determined with the help of software is depicted in surface response curves as shown in Figure 4. A checkpoint batch (TCP) was prepared at X_1 = 0.29 level and X_2 = 0.99 level at which Yield% and DE_{120}% was 68.4±0.7 and 40.2±0.5, respectively. The optimized batch (TCP) depicted the expected results. The desirability of the optimized batch was 0.917003.

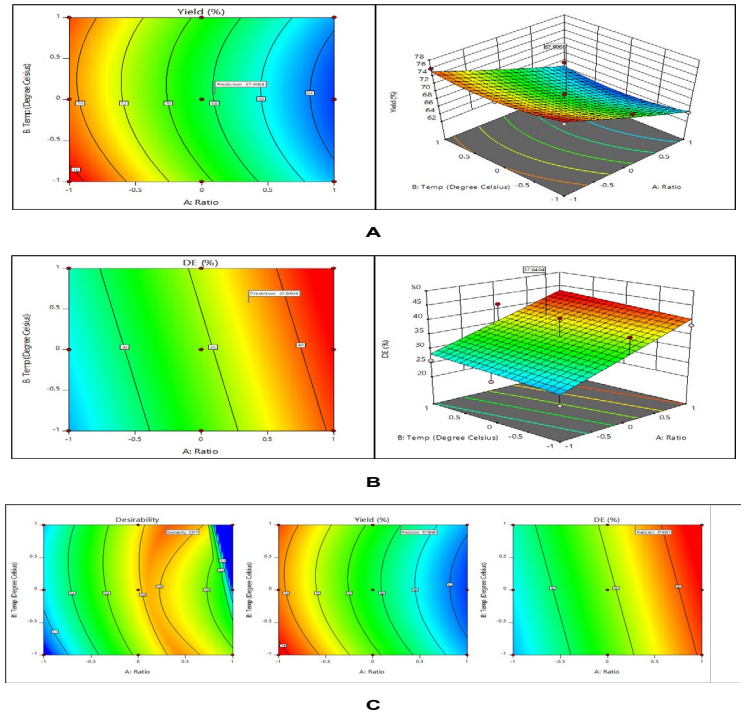

Figure 4. Response surface plots of (A) percentage yield, (B) percentage dissolution efficiency, and all response surface plots of (C) spray-dried solid dispersions using optimization software

From the optimization conducted, it has shown optimized data in a coding form. Based on the input data, it showed a 0.29 and 0.99 coded response to X_1 and X_2 in the 3^2 full factorial design. The responses were decoded and found to be 1:2.3 and 109.9, respectively. In other words, for maximum %Yield and $\%DE_{120}$, the drug: polymer ratio and spray drier inlet temperature should be 1:2.3 and 109.9°C, respectively. To evaluate the prediction capability of the models and to verify the optimization process, spray-dried solid dispersions were prepared based on optimal process variable settings.

Characterization of optimized formulations

IR spectral analysis

Initially, the FTIR spectrum of a drug can be used to determine the functional groups in that compound. As shown in Figure 5, FTIR spectra of pure bosentan showed characteristic peaks at 3629.42 cm^{-1} for O-H stretch, 3447.55 cm^{-1} for N-H stretch, 2961.29 cm^{-1} for C-H stretch aliphatic, 1577.83 cm^{-1} for N-H bend, 1341.95 cm^{-1} for S=O, and 1170.85 cm^{-1} for sulfonamide. The FTIR spectra of formulations showed a slight shift in the peaks 2883 cm^{-1} and 2880 cm^{-1} (C-H stretch aliphatic) for FL10 and FS10, respectively, without any other significant changes. This could be due to possible intermolecular hydrogen bonding in the formulations (Dangre, 2017). The IR spectrum of poloxamer 188 is characterized by principal absorption peaks at 2885.13 cm^{-1} (C-H stretch aliphatic), 1342.56 cm^{-1} (in-plane O-H bend), and 1108.31 cm^{-1} (C-O stretch). Characteristic peaks of bosentan monohydrate and poloxamer 188 seemed to be preserved in the prepared solid dispersion, which proved that there was no chemical interaction between the drug and the excipient.

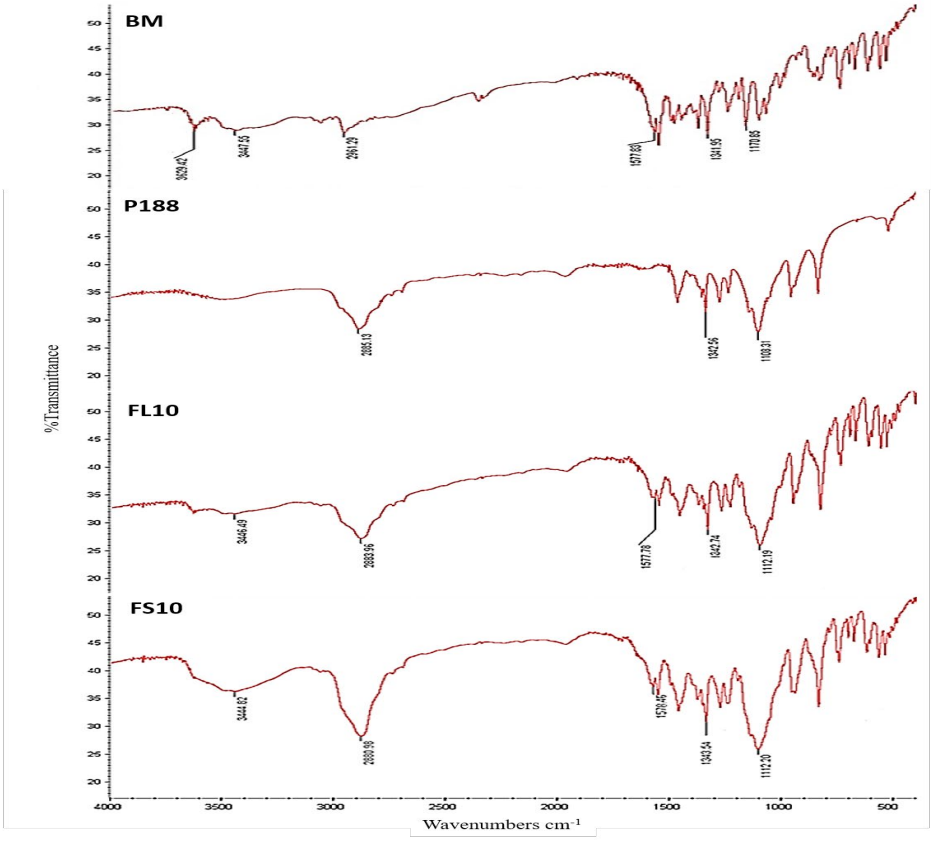

Figure 5. FTIR spectral analysis of bosentan monohydrate (BM), poloxamer 188 (P188), and optimized formulations FL10 and FS10

Particle size and size distribution analysis

The particle size of the optimized solid dispersions was determined using a particle size analyzer. The samples were dissolved in triple distilled water and subjected to particle size analysis. This method also depicts the polydispersity index (PDI), which is a measure of uniformity in size distribution. As shown in Figure 6, the average particle size and the PDI of FL10 were 450.9 nm and 0.401, respectively. And in the case of the FS10, the average particle size and the PDI were found to be 550.8 nm and 0.590, respectively.

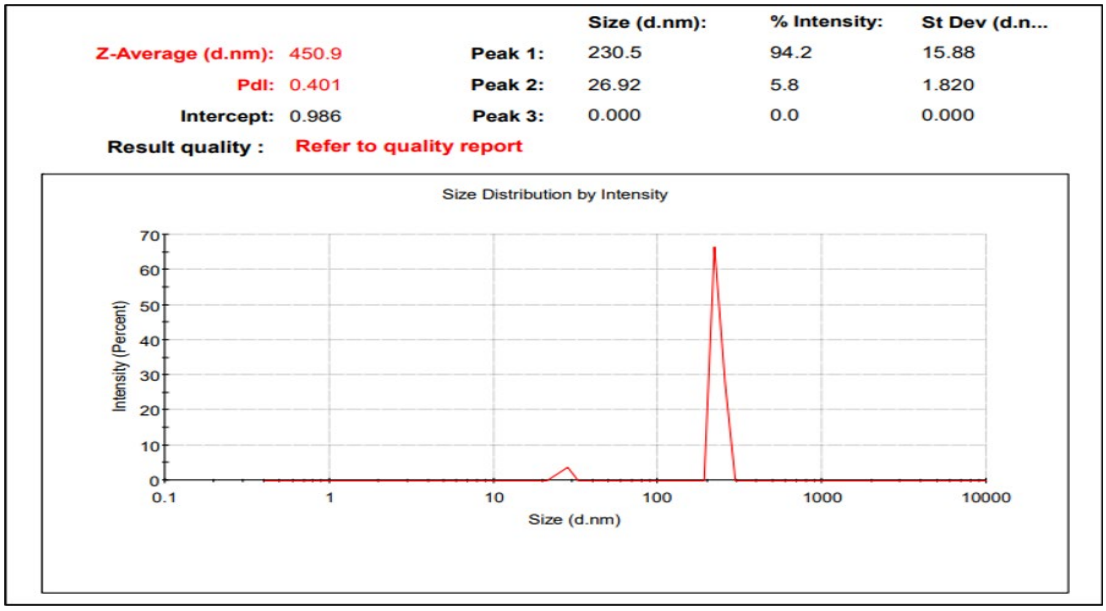

Figure 6. Particle size and size distribution of solid dispersions (A) FL10 and (B) FS10

Differential scanning calorimetry (DSC)

DSC curves of pure drug, P188, and prepared solid dispersions (FL10 and FS10) with P188 are shown in Figure 7. For pure BM, a sharp endothermic peak is observed at 128.64°C, characterizing the melting point of BM, which indicates that the pure drug was in crystalline form. P188 showed a melting endothermic peak at 58.07°C. Upon the formation of solid dispersions of drug with P188, there was a disappearance of the drug melting endotherm in the solid dispersions, which could be due to the amorphous form of BM in the solid dispersions. But in both solid dispersions, the sharp peak corresponding to polymer remained and was at a slightly lower temperature than that of pure P188 (58.07°C). It might be due to the reason that drug molecules get dispersed in the P188 matrix of the solid dispersions and the thermal property was changed, or it might be due to the formation of eutectic mixtures in solid dispersions leading to the depression of melting point (Zhai, 2017). Further, to deep root these findings, XRD of pure drug and its solid dispersions was carried out.

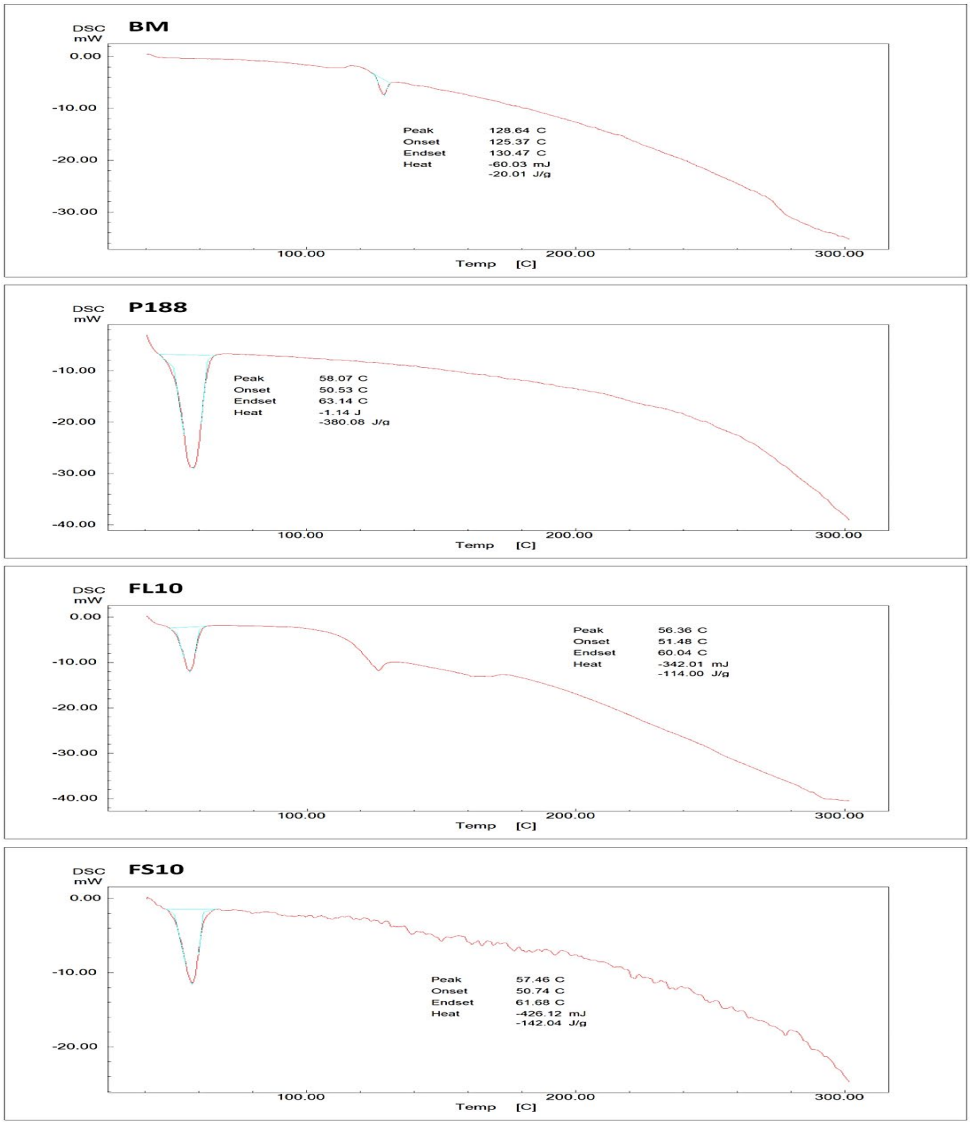

Figure 7. DSC of bosentan monohydrate (BM), poloxamer 188 (P188), and optimized solid dispersions prepared with poloxamer 188 by lyophilization and spray drying methods (FL10 and FS10)

X-ray diffraction (XRD)

X-ray diffraction patterns were used to confirm the crystalline nature of the drug. As shown in Figure 8, pure BM exhibited distinct sharp peaks at 2θ diffraction angles of 18.42°, 9.1°, 22.5°, and 16.48°, which were intense and displayed sharp intensities of 7535, 4153, 4090, and 3495, respectively, indicating its crystalline nature. In comparison, P188 showed sharp crystalline peaks at 23.18° and 19°. Both solid dispersions prepared with P188 exhibited the disappearance of some high-intensity drug peaks and a reduction in the intensity of polymer peaks. Compared to FS10, the lyophilized FL10 solid dispersion showed low-intensity peaks of corresponding drug and polymer. Hence, there was a reduction in crystallinity in both solid dispersions prepared using both methods. The disappearance or decrease in intensity of the peaks at the same diffraction angles in solid dispersions prepared with P188 indicates that BM may have undergone a transition from crystalline to amorphous form or crystallinity was reduced. Moreover, the high-intensity peaks of P188 indicating its crystalline nature also got diminished.

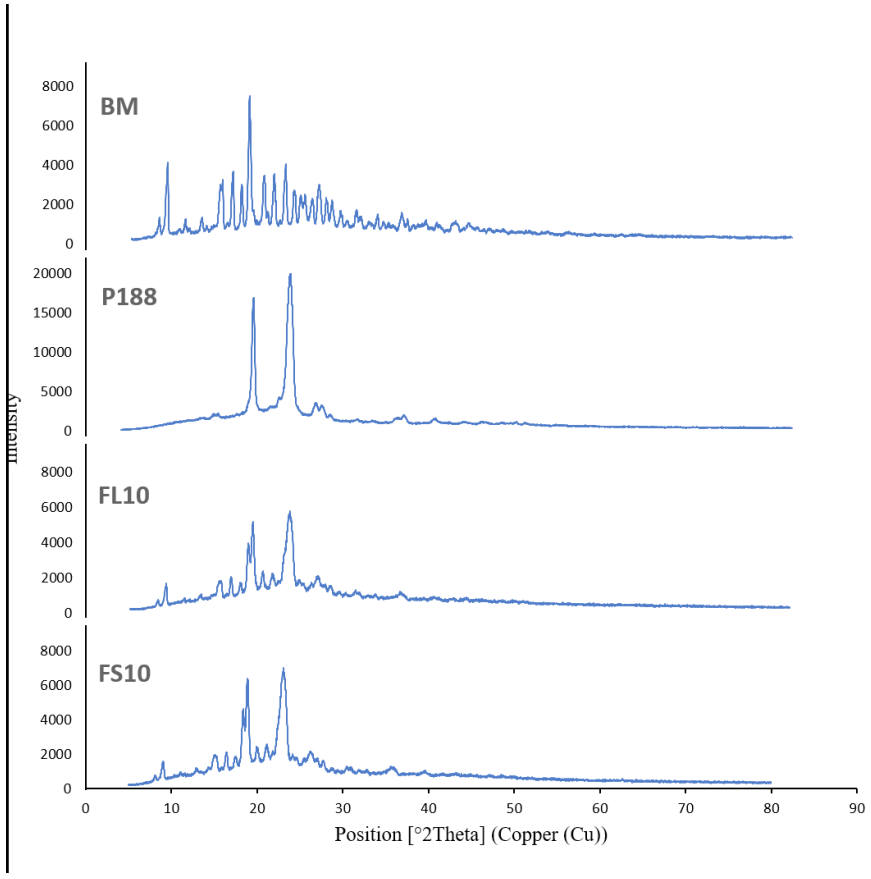

Figure 8. XRD of bosentan monohydrate (BM), poloxamer 188 (P188), and optimized solid dispersions prepared with poloxamer 188 by lyophilization and spray drying methods (FL10 and FS10)

Scanning electron microscopy (SEM)

The SEM photomicrographs of pure BM, P188, and optimized solid dispersions prepared using lyophilization and spray drying methods (FL10 and FS10) are shown in Figure 9. The pure drug appeared as crystals, whereas P188 and solid dispersions revealed amorphous particles. Solid dispersion prepared with P188 using lyophilization technique (FL10) showed the formation of a porous and fluffy product that increases the surface area and in turn, the surface free energy, resulting in higher solubility and dissolution. For further analysis of FL10 and to confirm its amorphous state, it was subjected to SEM analysis at higher magnification as shown in Figure 10.

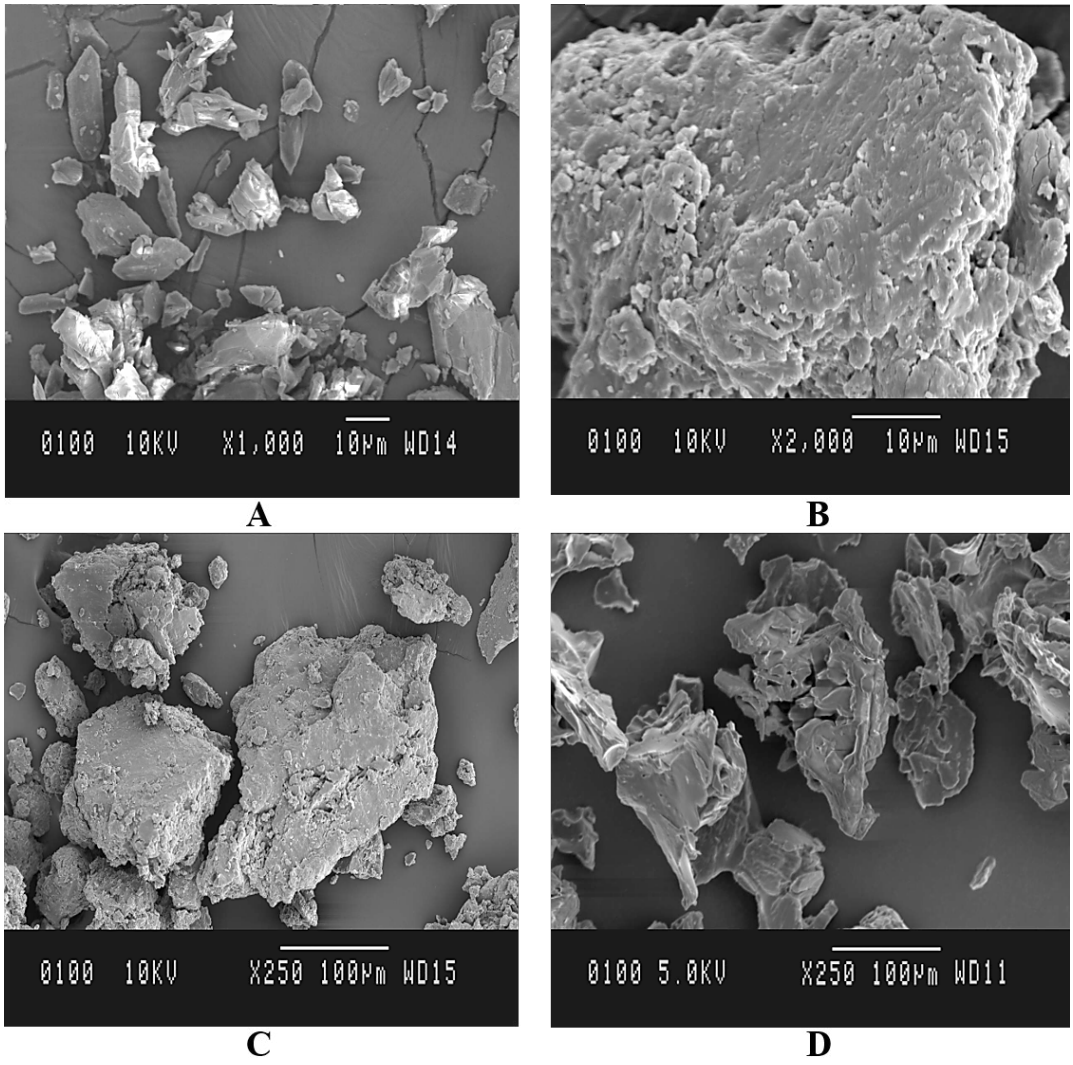

Figure 9. SEM of (A) pure bosentan monohydrate, (B) poloxamer 188, (C) solid dispersion FL10, and (D) solid dispersion FS10

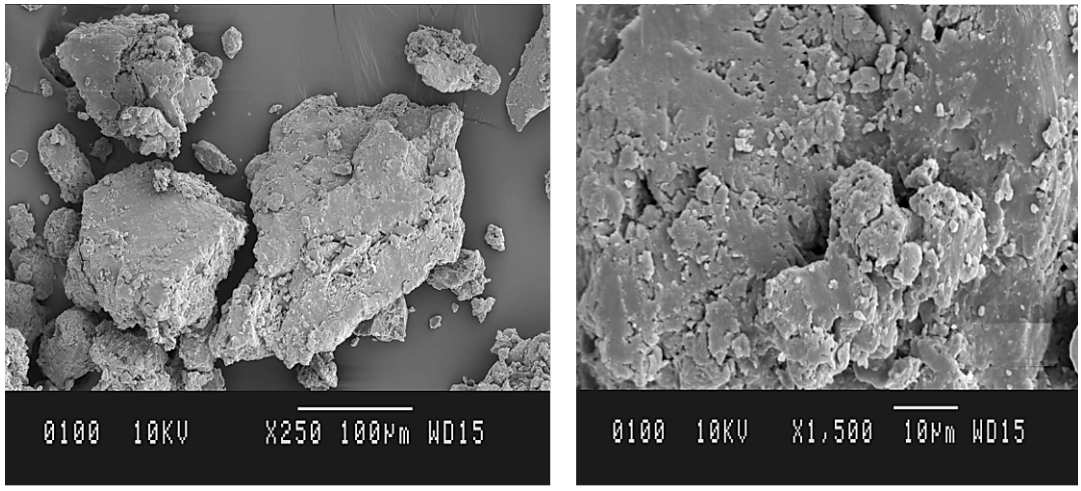

Figure 10. SEM of optimized solid dispersion formulation FL10 in magnifications of (A) 250x and (B) 1500x

Transmission electron microscopy (TEM)

As FL10 showed improved properties in previous tests, A TEM of FL10 was carried out to further determine its particle shape and particle size. As shown in Figure 11, the particle size of optimized solid dispersion FL10 prepared with P188 was found to be 505.68 nm. The reduced particle size of solid dispersion FL10 confirms why lyophilized solid dispersions were showed improved solubility and dissolution rate as compared to the solid dispersion prepared using the spray drying method. Additionally, FL10 demonstrated an acceptable shape, indicating that it may exhibit good flow properties.

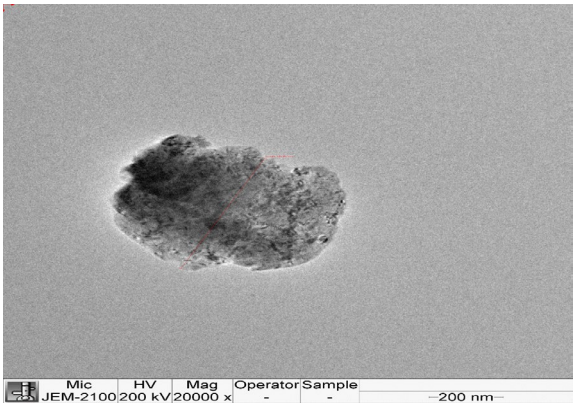

Figure 11. TEM of solid dispersion formulation FL10 prepared with poloxamer 188

Stability study of optimized solid dispersion

An accelerated stability study of optimized solid dispersion FL10 at room temperature was conducted for 3 months and any physical changes were analyzed. The results of stability studies of optimized solid dispersions of BM are shown in Table 8. There were no changes observed in the physical appearance of the solid dispersions during the storage period of 3 months. The drug content also showed no significant difference. Two characterization studies, i.e., XRD and particle size analysis were conducted to determine any change in the amorphous nature of prepared dispersions during storage. In the case of XRD studies of optimized dispersion, no sharp endothermic peaks were observed and the high-intensity peaks of the corresponding drug were absent. No significant changes were observed in both analyses up to 3 months of storage.

Table 8. Stability study of optimized solid dispersion FL10

Formulation code	Characterization	Days				
		0	30	45	60	90
FL10	Physical appearance	No change	No change	No change	No change	No change
	%DC	98.9 ±1.1	97.8 ±1.3	97.08 ±2.4	96.89 ±1.3	96.51 ±1.6
	XRD	-	-	-	-	No significant changes in the intensity of peaks
	Particle size analysis	-	-	-	-	No significant change in particle size

Data are expressed as mean ±SD (n=3); %DC: Percentage drug content, XRD: X-ray diffraction.

CONCLUSION

Solid dispersions of bosentan monohydrate were prepared to enhance its aqueous solubility and determine the effect of using lyophilization or spray drying method on solubility enhancement. Solid dispersions were prepared by using poloxamer 188 as the carrier. It was found that the particle size and solubility of the dispersions were significantly affected by the type of method used. According to the analytical study results, the lyophilization technique was more effective at preparing solid dispersions. The optimized solid dispersion prepared with poloxamer 188 by the lyophilization technique (FL10) showed a smaller particle size and was subjected to different characterization studies. The findings of this study substantiate the notion that solid dispersion reduces the particle size and crystallinity of a drug while increasing its aqueous solubility. The study also demonstrates that the lyophilization technique is better as compared to spray drying due to improved solubility, dissolution, reduced particle size, a significant reduction in crystallinity as indicated by XRD and formation of more amorphous product as indicated by SEM. Hence, it can be concluded that lyophilization technique shows superior results as compared to spray drying in the preparation of solid dispersions.

CONFLICTS OF INTEREST

No conflict of interest is declared by the authors. The authors alone are responsible for the content and writing of the paper.

AUTHOR CONTRIBUTION STATEMENT

Research hypothesis (Kaur L., Singh G., Chemban S.A.). Experimentation and data collection (Chemban S.A., Kaur A., Singh L). Draft of the text (Chemban S.A., Dhawan R.K., Kaur L.) Interpretation of the data and use of software (Kaur L., Singh G., Dhawan R.K., Chemban S.A., Singh L.). Text reviewed (Kaur L., Singh G., Dhawan R.K.). Statistical analysis (Kaur L., Singh G., Chemban S.A.). Literature research (Chemban S.A., Singh L., Singh G.). Writing, review & editing (Kaur L., Singh G., Dhawan R.K., Singh L.)

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RESEARCH ARTICLE

Potential Use of Breadfruit (*Artocarpus altilis*) Leaf Extract to Recover Hepatic and Renal Damage in Alloxan-Induced Diabetic Rats

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Potential Use of Breadfruit (Artocarpus altilis) Leaf Extract to Recover Hepatic and Renal Damage in Alloxan-Induced Diabetic Rats

SUMMARY

The antihyperglycemic effect of breadfruit leaf (Artocarpus altilis) extract has been demonstrated in a preclinical study using an alloxan-induced diabetic model. This study aimed to examine whether breadfruit leaf extract also ameliorated liver and kidney injury in alloxan-induced diabetic rats. Male Wistar rats (n=35) were used in the study. All other animals except control group (group I, n=5) were injected with alloxan (155 mg/kg body weight). After 3 days, the hyperglycemic rats with blood glucose >200 mg/dl were divided into 4 treatment groups: placebo (alloxan group), Breadfruit Leaf (BL) extract 100 mg/kg, BL extract 200 mg/kg, and BL extract 400 mg/kg. Treatments were administered daily for 14 days, and blood samples were drawn at baseline, after alloxan injection, and following treatments to obtain serum glutamic pyruvic transaminase (SGPT) and creatinine levels. Alloxan was found to cause a significant increase in rat blood glucose, SGPT, and creatinine levels three days post alloxan injection (P<0.01). After treatment, rats that received 200 mg/kg and 400 mg/kg BL extracts had significantly lower SGPT levels compared to those treated with placebo alone (P<0.05). Liver histological damage was also significantly alleviated, especially with the 400 mg/kg dose of BL extract. Although serum creatinine level was restored, alloxan-induced tubular degeneration in renal tissue was still evident. In conclusion, BL extract at a dose of 400 mg/kg improved alloxan-induced liver dysfunction and tissue damage but was less effective at alleviating kidney damage. This result may support the use of breadfruit leaf extract as herbal drug with a hepatoprotective effect.

Key Words: *Breadfruit leaf, Artocarpus altilis, diabetic rats, alloxan, liver damage, kidney damage*

Alloxan Nedenli Diyabetik Sıçanlarda Hepatik ve Renal Hasarı İyileştirmek İçin Ekmek Meyvesi (Artocarpus altilis) Yaprak Ekstresinin Potansiyel Kullanımı

ÖZ

Ekmek meyvesi yaprağı (Artocarpus altilis) ekstresinin antihiperglisemik etkisi, alloksan nedenli diyabet modeli kullanılarak yapılan in vivo bir çalışmada gösterilmiştir. Bu çalışma, ekmek meyvesi yaprak ekstresinin, alloksan nedenli diyabetik sıçanlarda karaciğer ve böbrek hasarını iyileştirip iyileştirmediğini incelemeyi amaçlamıştır. Çalışmada erkek Wistar sıçanları (n=35) kullanılmıştır. Kontrol grubu (grup I, n=5) dışındaki tüm diğer hayvanlara alloksan (155 mg/kg vücut ağırlığı) enjekte edilmiştir. Üç gün sonra, kan şekeri >200 mg/dl olan hiperglisemik sıçanlar 4 tedavi grubuna ayrılmıştır: plasebo (alloksan grubu), ekmek meyvesi yaprak (BL) ekstresi 100 mg/kg, BL ekstresi 200 mg/kg; ve BL ekstresi 400 mg/kg. Tedaviler 14 gün boyunca günlük olarak uygulanmış ve başlangıçta, alloksan enjeksiyonundan sonra ve tedavileri takiben serum glutamik piruvik transaminaz (SGPT) ve kreatinin düzeylerini belirlemek için kan örnekleri alınmıştır. Alloksanın, enjeksiyondan 3 gün sonra sıçan kan şekeri, SGPT ve kreatinin seviyelerinde önemli bir artışa neden olduğu bulunmuştur (P<0.01). Tedaviden sonra, 200 mg/kg ve 400 mg/kg BL ekstreleri uygulanan sıçanların, tek başına plasebo ile tedavi edilenlere kıyasla önemli ölçüde daha düşük SGPT seviyelerine sahip olduğu görülmüştür. (P<0.05). Karaciğer histolojik hasarı da, özellikle 400 mg/kg dozda BL ekstresi ile önemli ölçüde azalmıştır. Serum kreatinin düzeyi eski haline gelmesine rağmen, böbrek dokusunda alloksan nedenli tübüler dejenerasyonun hala belirgin olduğu gözlenmiştir. Sonuç olarak, 400 mg/kg vücut ağırlığı dozunda uygulanan BL ekstresi, alloksan nedenli karaciğer fonksiyon bozukluğunu ve doku hasarını iyileştirmiş, ancak böbrek hasarını iyileştirmede daha az etkili bulunmuştur. Bu sonuç, hepatoprotektif etkili bir bitkisel ilaç olarak ekmek meyvesi yaprağı ekstresinin kullanımını destekleyebilir.

Anahtar Kelimeler: *Ekmek meyvesi yaprağı, Artocarpus altilis, diyabetik sıçanlar, alloksan, karaciğer hasarı, böbrek hasarı.*

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INTRODUCTION

The breadfruit plant (*Artocarpus altilis* (Parkinson) Fosberg) is a tropical plant that belongs to the family Moraceae (Akanbi, et al., 2009). The yellowed breadfruit leaves have been used as tea to reduce blood pressure and asthma in the West Indian region. The tea is also known for its therapeutic benefit to control diabetes, which may be derived from its complex organic acid contents (Ragone, 2018). The mechanism of antidiabetic effect of *A. altilis* aqueous extract is believed to be associated with the inhibition of carbohydrate metabolizing enzymes and the stimulation of glucose cellular uptake (Sairam & Urooj, 2012). The aqueous extract of *A. altilis* leaves is safe to use at the dose up to 2000 mg/kg body weight (Sairam & Urooj, 2014).

The therapeutic effects of *A. altilis* leaf ethanolic extract have been studied in diabetic animal models. It has been shown that the aqueous extract of *A. altilis* leaf can reduce blood glucose levels in alloxan-induced diabetic mice and rats (Thubasni, et al., 2012; Djabir, et al., 2021). In addition to the anti-hyperglycemic effect, the ethanolic extract of breadfruit leaves at a dose of 400 mg/kg body weight (BW) has been shown to improve insulin expression in pancreatic beta cells (Indrowati, et al., 2017) and reduce histopathological injury in the pancreatic tissue (Sari, et al., 2020). It is believed that the antioxidant constituents of *A. altilis* significantly contribute to its therapeutic effects on diabetes mellitus since its pathogenesis is predominantly triggered by free radicals and oxidative stress (Ceriello & Motz, 2004).

Many of the chemical compounds of *A. altilis* leaves have been identified years ago. These include tannins, phenols, glycosides, saponins, steroids, terpenoids, and anthraquinones (Graham & De Bravo, 1981). More recently, Sikarwar and co-workers (2014) have listed a range of specific phytochemical constituents of *A. altilis*, including artocarpetin, cycloartinone, cyclogeracommunin, cycloartenyl acetate, cyclocommunol, norartocarpetin, and oxydihydroartocarpesin (Sikarwar, et al., 2014b). These phytochemicals are believed to mediate a range of biological activities, including antioxidant and anti-hyperglycemic activities (Sikarwar, et al., 2014a).

One of the diabetogenic agents that are popularly used to induce diabetes mellitus in animals is alloxan (2,4,5,6-tetraoxypyrimidine). The molecule structure of alloxan that resembles glucose molecules enables its uptake through glucose transport GLUT-2 in beta cells of the pancreas. This facilitates the selective entry of alloxan into beta cells of the pancreas and ultimately damages these insulin-producing cells. As a result, the animals injected with alloxan will experience a decrease in insulin production, leading to hyperglycemia (Ighodaro, et al., 2017).

Although alloxan was initially thought to merely damage the pancreatic beta cells (Gorus, et al., 1982), a number of studies have shown that alloxan injection is not only toxic to pancreatic beta cells but also toxic to other organs expressing GLUT-2, including liver hepatocytes and kidney tubular cells (Gargouri, et al., 2016; Terayama, et al., 2016). Thus, it is often found that alloxan-induced diabetic animals also experience liver and kidney dysfunction. Since alloxan hydrogen toxicity is mediated by reactive oxygen species, including superoxides, peroxides, and radical hydroxyl (Lenzen, 2008), it is believed that plant extract that is rich in antioxidant compounds may reduce the oxidative damage induced by alloxan. Therefore, this study aimed to explore the potential use of *A. altilis* leaf extract to reduce hepatic and renal injury in diabetic rats induced by alloxan.

MATERIAL AND METHODS

Chemicals preparation

Alloxan monohydrate (Sigma Aldrich, Singapore) and other chemicals, including 70% ethanol, were purchased through official chemical distributors in Makassar, Indonesia. Reagents for Glutamic-Pyruvic Transaminase (GPT (ASAT) IFCC mod.liquiUV) and Creatinine (creatinine liquicolor) measurements were purchased from HUMAN Diagnostic Worldwide (Germany).

Breadfruit leaf collection

Breadfruit (*Artocarpus altilis* (Parkinson) Fosberg) leaves were collected at 8-10 am in Timbuseng Village, Patallassang District, Gowa Regency, South Sulawesi in July 2019. The herbarium specimen was stored in Pharmacognosy Laboratory, Faculty of Pharmacy, Hasanuddin University, Indonesia. The plant was authenticated and confirmed by Dr. A. Mu'Nisa from the Laboratory of Biology, State University of Makassar, Indonesia (No. 096/SKAP/LAB.BIOLOGI/VII/2019)

Breadfruit leaf extract preparation

The collected leaves are thoroughly washed with running water and sorted from foreign materials. The leaves were then washed, dried, and cut into simple 0.5 cm pieces before being macerated with 70% ethanol (1:10 ratio) for five days. Ethanol (70%) was chosen as the maceration solvent to allow optimal extraction of the phenolic compounds of *A. altilis* (Sao Mai, 2015). The maceration process was protected from sunlight. The resulting ethanolic extract was concentrated to dryness using a rotary evaporator (Heidolp®) and stored at room temperature (25°C) in a vacuum desiccator to remove the extra solvent. Prior to administration, the extract was prepared in 1% sodium carboxymethyl cellulose (Na CMC) suspension to facilitate extract administration in animals.

Animal preparation

Thirty-five 12-week-old (180-300 g) male Wistar rats were obtained from a rodent breeding facility (UD Wistar, Yogyakarta, Indonesia) and transferred to the laboratory where the experiment was conducted. Rats were housed in plastic cages with wood-shaving bedding in the laboratory with 12-hour light and dark cycle. The animals had free access to food and water. Animals were adapted at least 14 days before the start of the experiment. All animal protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals. The experiment received ethical approval from the Institutional Animal Ethics Committee of the Faculty of Medicine, Hasanuddin University, with the ethical number UH19050277.

Experimental protocols

Before starting any treatment, rats were anesthetized by placing the rat one by one in a chamber (2200 cm^3) filled with 0.5 ml ether-impregnated cotton balls until the rat was fully anesthetized. Blood samples were drawn from all rats to obtain baseline data. Blood samples (3 ml) were taken from the lateral vein and placed in blood collection tubes (BD vacutainer®) containing EDTA.

Except for healthy controls (n=5, Group I), all other rats (n=30) were injected intraperitoneally (i.p) with alloxan at a dose of 155 mg/kg body weight (BW) to induce diabetes mellitus. Approximately 10 minutes after alloxan injection, 5% glucose solution (2 ml/200 g BW) was administered orally to prevent acute hypoglycemia. Blood glucose level measurements were made after daily injection of alloxan using a digital glucometer (Nesco®). After 72 hours (three days) of alloxan injection, rats were anesthetized via inhalation with ether and 2 ml blood samples were taken to analyze blood glucose levels using Humalyzer 3500 (Human®). Only rats with blood glucose levels >200 mg/dl at 3 days after alloxan injection (n=20) were then assigned to receive one of the following treatments: Na CMC suspension without extract (Group II, n=5), Breadfruit leaf (BL) extract 100 mg/kg BW (Group III, n=5), Breadfruit leaf (BL) 200 mg/kg BW extract (Group IV, n=5), and Breadfruit leaf (BL) 400 mg/kg BW (Group V, n=5). Treatments were administered for 14 days before final blood samples were taken to measure blood glucose, serum glutamic pyruvic transaminase (SGPT), and creatinine levels after treatment. At the end of the experiment, all animals were euthanized by cervical dislocation and the livers and kidneys were collected for histopathological analysis.

Biomarker analysis

Blood samples were immediately centrifuged at 3000 rpm for 20 minutes to obtain serum and placed in the refrigerator (-20°C) until further analysis. Blood glucose, SGPT, and creatinine levels were analyzed using reagent kits for Humalyzer 3500 (Human®) according to the kits' instructions.

Histopathological analysis

Rat livers and kidneys were washed with phosphate buffer solution (PBS) before fixing with 10% formaldehyde. Tissue samples were processed in a tissue processor and prepared in paraffin blocks. Tissue blocks were sliced approximately 4-5 μm thick using a microtome, and then tissue sections were stained with standard Hematoxylin and Eosin (HE) staining. The presence of histopathological changes was analyzed under the light microscope by an anatomical pathologist blinded to the treatment groups. Levels of histopathological injuries were determined according to the area and intensity of necrosis, tissue degeneration, and inflammation using methods described in the Gibson-Corley, et al. (2013) study.

Statistical analysis

Obtained data such as blood glucose, SGPT, and creatinine levels were tested for normal distribution with the Kolmogorov-Smirnov test. To determine the significant difference for group treatments, repeated measured analysis of variance (ANOVA) statistical testing was performed at 95% confidence level, followed by post hoc Tukey's honestly significant difference (HSD) test.

RESULTS AND DISCUSSION

Rat blood glucose levels and body weight

Alloxan is a potent diabetogenic agent that can acutely induce hyperglycemia in rats 6 hours after i.p injection. In this study, the initial blood glucose level of rats ranged from 82-110 mg/dl. After three days from alloxan injection, rats' blood glucose levels rose to 261 - 372 mg/dl, which is 2-3 times of the blood glucose level of the healthy controls (Table 1).

There are three mechanisms by which alloxan induces hyperglycemia: 1) it selectively inhibits insulin secretion through glucokinase inhibition, 2) it stops the detection of sugar by beta cells, and 3) it induces the generation of reactive oxygen species (ROS), causing its selective necrosis in beta-pancreatic cells, leading to an insulin-dependent diabetes state (Lenzen, 2008). Two recent studies have reported the presence of inflammation and necrosis in pancreatic tissue of rats treated with alloxan (Sari et al., 2020), as well as shrinkage of pancreatic islets (Djabir et al., 2021). However, induction of hyperglycemia by alloxan has not always been 100% successful. Ten out of 30 animals (33%) did not experience a significant increase in blood glucose levels and were therefore excluded from this study.

Rats with blood glucose levels of >200 mg/dl were then randomly assigned to either receive placebo or BL extract treatments as presented in Table 1. In the alloxan group, which only received placebo, the level of blood glucose was constant above 300 mg/dl after 14 days of treatment. In contrast, a significant reduction of blood glucose level was found (from 372 ± 63.2 to 118 ± 26.1 mg/dl) in rats treated with BL extract at a dose of 400 mg/kg BW ($p<0.05$). Meanwhile, the lower doses (100 and 200 mg/kg BW) were not adequate to alleviate the alloxan-induced hyperglycemia. Hence, the blood glucose levels of those groups remained >200 mg/dl after 14 days of treatment (Table 1).

Table 1. Rat blood glucose levels and body weight before injection, after three days from alloxan injection, and following 14 days of treatment.

Treatment group	Blood glucose level (mg/dl)			Body weight (g)		
	Before injection	After injection	After treatment	Before injection	After injection	After treatment
Control (no alloxan)	104 ± 4.0	136 ± 22.5	116 ± 10.9	196 ± 11.5	201 ± 11.7	217 ± 16.0
Alloxan	107 ± 5.2	276 ± 26.6*	273 ± 41.9	224 ± 26.2	212 ± 26.7	204 ± 25.9
Alloxan + BL 100	103 ± 6.4	292 ± 43.7*	279 ± 43.2	236 ± 15.1	220 ± 21.3	215 ± 29.4
Alloxan + BL 200	107 ± 4.8	261 ± 25.3*	252 ± 69.5	234 ± 22.9	221 ± 25.5	219 ± 18.6
Alloxan + BL 400	111 ± 4.5	372 ± 63.2*	118 ± 26.1#	206 ± 3.3	197 ± 7.3	205 ± 17.2

*$p<0.05$ compared to blood glucose level before injection; #$p<0.05$ compared to blood glucose level after injection

Alongside hyperglycemia, weight loss is also one of the important features of diabetic rats. It is often that diabetic rats lose>10% of their body weight and are accompanied by lethargy and soft stool (Wang-Fischer & Garyantes, 2018). These symptoms were also observed in this study after rats receiving alloxan injection. The baseline body weights of rats ranged from 180-300 g then slightly decreased following alloxan injection. The average of weight loss of alloxan-treated groups was 9 g to 16 g after three days from the injection, while the control group gained weight as much as 5 g on average (Table 1). Indeed, following 14 days of treatments, the rat body weight continued to drop in 80% of rats in the alloxan group. On average the rats in alloxan group had lost 9% of their baseline body weight. Meanwhile, only 40% of rats treated with BL extract 400 mg/kg experienced further weight loss and only lost 0.5% of their body weight. This may indicate an improvement of metabolic function in rats that received BL extract 400 mg/kg.

Liver function and tissue structure post alloxan injection and following treatments

The SGPT levels of rats before, after and 14 days after alloxan injection were presented in Figure 1. Alloxan has been shown to significantly increase the level of SGPT after three days of injection. The increase in the mean level of SGPT in alloxan group, alloxan + BL 100, alloxan + BL 200, and alloxan + BL 400 were 62%; 100%; 34%; 113%, respectively. Indeed, the SGPT level of rats in the alloxan group continued to rise to >200 mg/dl after 14 days of treatment, which was five times higher than baseline (p<0.05).

The result of this study shows that breadfruit leaf (BL) extract treatment can preserve the liver function of alloxan-injected rats, as the use of BL extract can prevent the increase of SGPT levels (Figure 1). The most effective dose was found to be 200 and 400 mg/kg rat body weight. With the administration of BL extract, the SGPT levels returned to normal despite the initial increase after alloxan injection (p< 0.05). The post-treatment SGPT level was also lower in the alloxan + BL extract 100 mg/kg group compared to the alloxan group. Still, the reduction in SGPT was less noticeable with 100 mg/kg BL extract compared with the higher doses.

Increased SGPT level is a specific indicator of liver dysfunction and liver injury, as this enzyme is normally contained in the hepatocytes (Djabir et al., 2020). The presence of liver injury following alloxan injection may be triggered by the direct effect of ROS generation or indirectly by uncontrolled hyperglycemia (Gargouri et al., 2016). Increased ROS production eventually leads to hepatocyte cell degeneration and cellular necrosis (Lucchesi et al., 2015), resulting in increased GPT release from the cytoplasm of hepatocytes into the circulation.

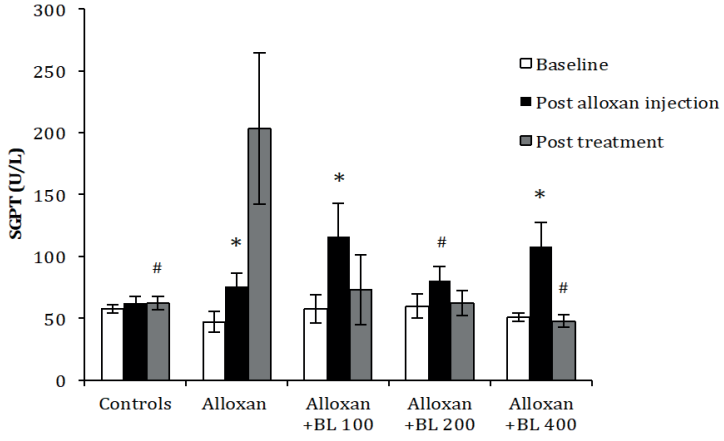

Figure 1. SGPT level of treated rats at baseline, post alloxan injection and post 14 days of treatments.

*shows p<0.05 compared to the baseline level. #shows p<0.05 compared to the alloxan group post-treatment.

In this study, liver histology of all rats injected with alloxan was found to show fatty and hydropic degeneration. Still, among all groups, the alloxan group experienced the most extensive necrosis in their livers (Figures 2B and 2C). Oral administration of breadfruit leaf ethanol extract appeared to improve hepatocyte cell structure. At a lower dose (100 mg/kg body weight), rat liver presented hydropic degeneration affecting 50% of liver tissues observed (Figure 2D). As for the 200 mg/kg (Figure 2E) and 400 mg/kg BW (Figure 2F) groups, some hepatocytes still showed signs of hydropic change but had significantly fewer necrotic cells overall compared to the alloxan group. Characteristics of liver injury in rats treated with alloxan can range from inflammation to necrosis (Bilal et al., 2016). The improvement in liver structure seen with BL extract might be enhanced by potent antioxidants and high phenolic content in breadfruit leaves which stabilize the ROS produced by alloxan (Leng et al., 2018).

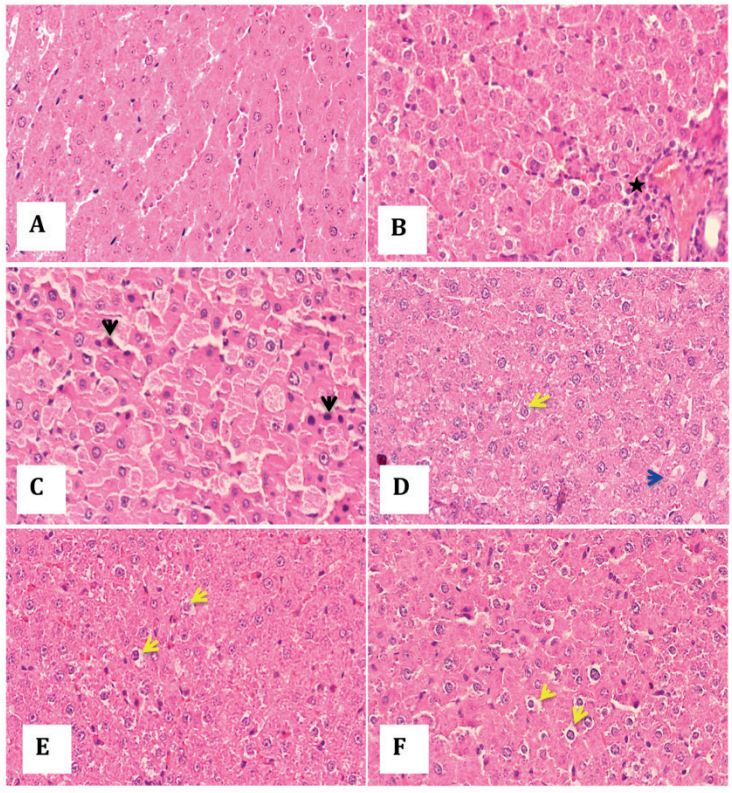

Figure 2. The photomicrograph of liver tissues of rats following 14 days of treatments (H&E stained, 400X magnification). **A**) The control group showed a normal architecture of hepatocytes and portal triad. **B**) Alloxan group showed an infiltration of inflammatory cells in liver tissue (*). **C**) Diffuse necrotic area and necrotic cells were found scattered in Alloxan-treated rats (black arrow). **D**) The liver from the rat treated with Alloxan + BL extract 100 mg/kg showed hydropic degeneration (yellow arrow) and dilated sinusoid (blue arrow). **E**) Rat's liver treated with Alloxan + BL extract 200 mg/kg mostly showed hydropic changes (yellow arrow). **F**) Rat's liver treated with Alloxan + BL extract 400 mg/kg showed hydropic degeneration (yellow arrow).

Renal function and tissue structure post alloxan injection and following treatments

Figure 3 depicts the serum creatinine levels at baseline and those initially treated with alloxan three days after alloxan injection and 14 days after treatment. After the injection of alloxan, creatinine levels were significantly elevated in all rats injected with alloxan compared to normal controls ($p< 0.05$). Alloxan-treated rats experienced at least a 120% rise

in creatinine on day three after alloxan injection. It is believed that elevated creatinine levels are due to the accumulation of glycogen in the distal tubules of the kidneys during persistent hyperglycemia (Terayama et al., 2016). Regardless of the treatment given, the creatinine level of alloxan-treated rats was simultaneously reduced by 50% (Figure 3). Spontaneous recovery of creatinine levels was also observed by another study after 30 days of alloxan injection in diabetic rabbits (Ahmad et al., 2014).

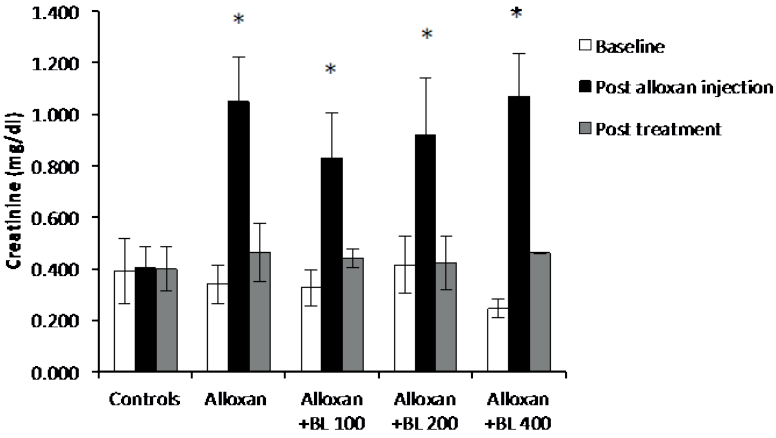

Figure 3. Serum creatinine level of rats at baseline, post alloxan injection, and post 14 days of treatments. *shows $p<0.05$ compared to the baseline level.

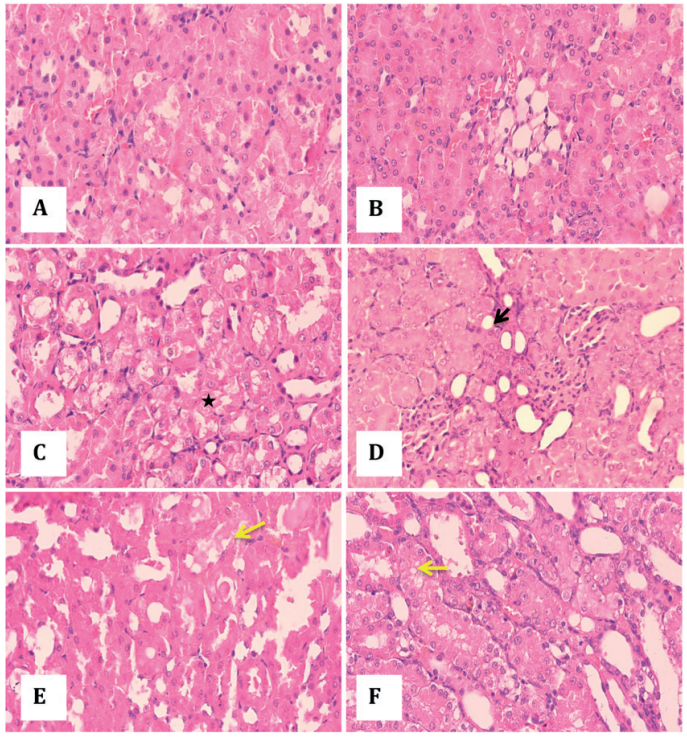

Figure 4. The photomicrograph of renal tissues of rats following 14 days of treatments (H&E stained, 400X magnification). **A)** The control group showed normal architecture of renal tubules. **B)** Alloxan group showed substantial lipid degeneration in the tubules. **C)** Alloxan group experienced degeneration in the renal tubule (*). **D)** Rat treated with Alloxan + BL extract 100 mg/kg showed lipid degeneration (black arrow). **E)** Rat treated with Alloxan + BL extract 200 mg/kg showed hydropic degeneration (yellow arrow). **F)** Rat treated with Alloxan + BL extract 400 mg/kg showed hydropic degeneration (yellow arrow).

The increase in creatinine is more likely to reflect acute renal dysfunction rather than permanent kidney damage as it returned to baseline 14 days after injection. Interestingly, renal histological changes were still prominent in placebo-only treated rats (alloxan group, Figures 4B and 4C) (25% to 50% of the field of view at 400X magnification). In this group, both fatty degeneration and hydropic changes were observed in the renal tubules. Application of the BL extract seemed to slightly reduce the severity of kidney injury since the damage was not as intense as in the alloxan group. Although some lipid degeneration was still observed with BL extract treatment at 100 mg/kg (Figure 4D), BL extract 200 mg/kg and 400 mg/kg treatment had less intense injury with scattered hydropic degeneration (Figures 4E and 4F). The discrepancy between the corrected serum creatinine level and the presence of histopathological changes in the renal tubules of alloxan-induced rats suggests that serum creatinine may not be the best biomarker for tubular damage (Chu et al., 2016). Further studies should include other biomarkers to quantify the extent of kidney injuries due to alloxan injection.

CONCLUSION

The injection of alloxan 155 mg/kg in rats led to hyperglycemia at three days post injection. Liver and kidney injuries were observed in the alloxan-induced diabetes model in rats. The administration of BL extracts at a dose of 200 mg/kg and 400 mg/kg were shown to reduce the SGPT level, indicating its potential role in improving alloxan-induced liver dysfunction. Breadfruit extract, especially at 400 mg/kg dose, significantly alleviated liver tissue injuries, and renal damage to a lesser extent. Due to the spontaneous recovery of serum creatinine level, it was difficult to assess the implication of BL extract in alloxan-induced renal dysfunction.

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CONFLICT OF INTEREST

The authors declared no conflict of interest

AUTHOR CONTRIBUTION STATEMENT

HS was responsible for conducting experiment, data acquisition and analysis, as well as writing the manuscript. YY as the project leader was responsible for designing the research protocols and concept, data interpretation, and manuscript revision. HH was responsible for sample extraction, biomarker analysis, and data editing. SL was responsible for data interpretation and revising the manuscript. HC was responsible for histopathological analysis result and interpretation

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RESEARCH ARTICLE

Design and Characterization of Fluconazole Loaded Elastic Liposome Based Gel for Treatment of Keratomycosis

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Design and Characterization of Fluconazole Loaded Elastic Liposome Based Gel for Treatment of Keratomycosis

SUMMARY

Fungal corneal ulcers, also known as keratomycosis, occur due to a breach in the corneal epithelium. According to WHO, it is the leading cause of blindness. The eye consists of a variety of different structures having different physiological functions that make it highly resistant to external substances, thus resulting in low bioavailability of drugs from most of the conventional dosage forms. To improve drug effectiveness, a series of research groups have tried a variety of strategies. The majority of these modifications provide some benefit over traditional dosage forms, but they have their own set of drawbacks. To overcome the side effects of the formulations mentioned above, Fluconazole-loaded elastic liposome-based gel was prepared. The elastic liposomes were prepared by rotary evaporation method using soya lecithin and sodium deoxycholate. The elastic liposomal suspension was then incorporated into optimised gelling agent (carbopol 934) to have sufficient contact time of the drug in the eye. The elastic liposome-based gel was then characterized for pH, drug content, rheological study, homogeneity and grittiness, in vitro release study, ex vivo permeation study, toxicity study, bio adhesion study and antifungal activity. The optimized formulation had pH 7.0 ± 0.01, drug content 98.5 ± 3.9%, viscosity 7217 ± 340 mPa.s, in vitro release 80.5± 0.32%, ex vivo permeation 72.27±0.65 % and the bio adhesion time of the optimized formulation was found to be significantly higher (p≤ 0.05) as compared to marketed gel. Antifungal activity of the optimized gel was also found to be significantly higher (p≤ 0.05) as compared to the marketed gel. The Fluconazole-loaded elastic liposome gel was prepared successfully and was found to be a good choice over conventional gel formulation for the treatment of keratomycosis.

Key Words: *Fungal corneal ulcers, Fluconazole, elastic liposomal gel, antifungal activity.*

Keratomikoz Tedavisi İçin Flukonazol Yüklü Elastik Lipozom Bazlı Jelin Tasarımı ve Karakterizasyonu

ÖZ

Keratomikoz olarak bilinen mantar kaynaklı kornea ülseri, korneal epitelyumunun yırtılması sonucu ortaya çıkar. WHO'ya göre keratomikoz körlüğün önde gelen nedenidir. Göz, dış faktörlere karşı oldukça dirençli olmasını sağlayan farklı fizyolojik fonksiyonlardaki farklı yapılardan oluşur, bu durum çoğu konvansiyonel dozaj formunun düşük ilaç biyoyararlanımı göstermesine neden olur. İlaç etkinliğini artırmak amacıyla birçok araştırma grubu çeşitli stratejiler denemişlerdir. Bu modifikasyonların büyük bir kısmı geleneksel dozaj formlarına kıyasla bazı yararlar sağlamışlardır ancak kendi dezavantajları vardır. Yukarıda bahsedilen formülasyonların yan etkilerinin üstesinden gelmek amacıyla, flukonazol yüklü elastik lipozom temelli jel hazırlanmıştır. Elastik lipozomlar, soya lesitin ve sodyum deoksikolat kullanılarak döner evaporasyon metodu ile hazırlanmışlardır. Sonrasında ilacın göz ile yeterli temasını sağlamak amacıyla elastik lipozomal süspansiyonlar optimize edilmiş jelleştirme ajanı (karbopol 934) ile birleştirilmiştir. Daha sonra elastik lipozom temelli jel pH, ilaç içeriği, reolojik çalışma, homojenite ve sürülebilirlik, in vitro salım çalışması, ex vivo permeasyon çalışması, toksisite çalışması, biyoadhezyon çalışması ve antifungal aktivite açısından karakterize edilmiştir. Optimize formülasyon 7,0 ± 0,01 pH değerine, %98,5 ± 3,9 ilaç içeriğine, 7217 ± 340 mPa.s viskoziteye, %80,5± 0,32 oranında in vitro salıma, %72,27±0,65 ex vivo permasyon değerine sahiptir ve biyoadhezyon süresi piyasa jeli ile karşılaştırıldığında anlamlı derecede (p≤ 0,05) yüksek bulunmuştur. Optimize jelin antifungal aktivitesi de ticati jel ile karşılaştırıldığında anlamlı derecede (p≤ 0,05) yüksek bulunmuştur. Flukonazol yüklü elastik lipozom jel başarılı bir şekilde hazırlanmış ve keratomikozis tedavisi için konvansiyonel jel formülasyonlarına göre ümit verici bir seçenek olarak bulunmuştur.

Anahtar Kelimeler: *Fungal kornea ülserleri, Flukonazol, elastik lipozomal jel, antifungal aktivite.*

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INTRODUCTION

Fungal corneal ulcers, also known as keratomycosis, are marked by stromal infiltration produced by fungi. Since the corneal epithelium acts as a barrier to microorganisms, these fungi induce infection when there is a breach in the epithelium (Chandra *et al*., 2013). The development of problems is sometimes preceded by a lack of suspicion and a delayed diagnosis, resulting in permanent eyesight loss or, worse, enucleation (Sharma *et al*., 2014).

The tropical climate and agriculture as significant occupation are the main causes of its high prevalence. Because of their increased vulnerability to occupational trauma, Indians are at an increased risk of keratomycosis. Annually, 1.5 to 2 million new cases of keratomycosis are reported, and keratomycosis is the leading cause of blindness, according to the World Health Organization (WHO). Keratomycosis is said to affects 44-47 percent of people in India (Chandra *et al*., 2013).

Candida, Aspergillus flavus, Fusarium species, etc., are a few microorganisms responsible for the onset of keratomycosis (Chandra *et al*., 2013).

The eye is an organ of vision and its physiology and anatomy make it a unique organ. The eye consists of various structures having different physiological functions that make it highly resistant to external substances (Palani *et al*., 2010), thus resulting in the low bioavailability of drugs from most of the conventional dosage forms (Yu *et al*., 2015). Another reason for the poor bioavailability of drugs from the conventional dosage forms is the pre-corneal loss variables such as insufficient residence time in the conjunctival sac, tear dynamics and non-productive absorption (Budai *et al*., 2007).

A series of research groups have tried a variety of strategies to improve drug effectiveness, including suspensions, ointments, inserts, hydrogels and polymeric micelles. The majority of these modifications provide some benefit over traditional dosage forms. Still, their drawbacks of obscured sight and patient non-compliance are the primary cause behind their lack of widespread acceptance (Yu *et al*., 2015).

To overcome the side effects associated with the formulations mentioned above, Fluconazole-loaded elastic liposomes were prepared for the treatment of keratomycosis. As reported in various literature, elastic liposomes have multiple advantages such as patient compliance due to painless administration of the drug, systemic side effects associated with oral formulations being bypassed, the drug is delivered to the target site directly (Hussain *et al*., 2017) and their ability to squeeze through channels 1/10th of their size and enter deeper tissues due to their elasticity (Benson, 2009), ensures a sustained release of drug with improved antifungal activity (Kumar *et al*., 2012).

However, due to their low viscosity, elastic liposomes generally need to be integrated into a suitable semi-solid dosage form for topical application and ample exposure time to drug in the eye. It is crucial to ensure product quality and production efficiency by controlling the flow qualities. By loading elastic liposome suspension in gel, the rheological properties of the suspension are improved (Dar *et al*., 2020) and the retention time of the formulation within the eye is increased (Sayeh *et al*., 2014).

In the current study, Fluconazole-loaded elastic liposomes were prepared to treat keratomycosis, using a Rotary evaporator. To improve the rheological properties, the elastic liposomal suspension was loaded in a gelling agent. The gel prepared was then evaluated for various parameters.

MATERIALS AND METHODS

Chemicals

Fluconazole was provided kindly by Ramson Remedies, Amritsar. Soya lecithin, sodium deoxycholate, carbopol 934 and sabouraud dextrose agar were procured from Hi-Media, Mumbai, India; chloroform was purchased from Molychem, Mumbai, India; methanol, sodium alginate and triethanolamine were procured from Merck life sciences Pvt. Ltd. Mumbai,

India. Carbopol 940 was purchased from Qualikems Fine Chem, Pvt. Ltd., Vadodra, India; HPMC was purchased from Thermo Fischer Scientific Pvt. Ltd., India. All the other chemicals and reagents were of analytical grade.

Preparation of elastic liposomes

The Rotary evaporation method was used for the development of drug-loaded elastic liposomes. Accurately weighed amounts of soya lecithin (280 mg), fluconazole (50 mg) and sodium deoxycholate (120 mg) were taken in a clean and dry round bottom flask and then the mixture was dissolved in an optimized quantity of organic solvent (Chloroform, 10 ml). The organic solvent was then evaporated under reduced pressure and high temperature (40° C) using a rotary evaporator. The film obtained at the base of the flask was then rehydrated with an aqueous phase (10 ml) (Kumar *et al*., 2012).

Preparation of elastic liposome-based gel

Selection of gelling agent

For the preparation of elastic liposome-based gel, different gelling agents (such as carbopol 934, carbopol 940, sodium alginate, and hydroxypropyl methylcellulose (HPMC)) were utilized at a concentration of 1%. In elastic liposomal suspension, the gelling agent was dispersed with constant stirring. In the case of carbopol 934 and carbopol 940, triethanolamine was utilized to alter the pH of the gel to 7 and was stirred continuously till a clear gel was obtained. The gels prepared were then characterized for physical appearance, homogeneity, grittiness and consistency (Kaur *et al*., 2018).

Formulation of elastic liposome-based gel

Four different concentrations (0.5%, 1%, 1.5%, and 2%) of the optimized gelling agent were taken and dispersed in elastic liposomal suspension. The mixture was stirred continuously. Triethanolamine was added under continuous stirring to adjust the pH of the mixture to 7 and was added until clear gel was obtained (Dar *et al*., 2020).

Evaluation of elastic liposome-based gel

Physical evaluation: Under normal daylight, the different gel formulations prepared were analyzed for physical changes (Abou *et al*., 2014). The experiment was done in triplicate.

***pH*:** The pH of different gel formulations was determined using a digital pH meter (Tawfeek *et al*., 2020). The experiment was done in triplicate.

Homogeneity and grittiness: By pressing a few milligrams of gel between the thumb and index finger, the homogeneity and grittiness of the prepared gel formulations were assessed (Abdellatif *et al*., 2016). The experiment was done in triplicate.

Drug content: By dissolving 1 g of properly weighed gel in methanol, the drug content of the gel was calculated. A UV spectrophotometer was used to measure absorbance at 261nm after appropriate dilution. The slope of the standard curve was used to determine the drug content (Phaldesai *et al*., 2014). The experiment was done in triplicate.

Rheological studies: Rheometer (Anton- Paar) was used for to analyze the rheological properties of the different gel formulations prepared. At 25° C, the viscosity of elastic liposome-based gel was measured using a spindle 25 (PP 25). The number of data points was set to 25, and the behaviour was set to ramp linear (Varges *et al*., 2019). The experiment was done in triplicate.

In vitro drug release study: Franz diffusion cell and treated dialysis membrane were used to determine the *in vitro* drug release of Fluconazole from different elastic liposome-based gels and drug solution gel (plain gel). Simulated tear fluid pH 7.4 (Sodium chloride = 0.670g, sodium bicarbonate = 0.2g, calcium chloride dehydrate = 0.008g, purified water = 100 ml) was used as a medium in the receptor chamber. The dialysis membrane was positioned amid the receptor chamber and the donor chamber. 1 g of accurately weighed gel was deposited on the dialysis membrane in the donor chamber. The whole assembly was

kept on the magnetic stirrer under constant stirring and the temperature of the assembly was maintained at 37°± 2°C. Samples were taken at preset time intervals and the amount withdrawn was restored with an equivalent amount of fresh media. The collected samples were filtered using a 0.45µm membrane filter prior to analysis.

UV spectrophotometer was used to determine the drug content in the samples withdrawn at a wavelength of 261 nm. A graph was plotted between the cumulative percent of drug released and time (El-Gizawy *et al*., 2020). The experiment was done in triplicate.

Ex vivo trans-corneal permeation study: Using Franz diffusion cell and excised goat corneal membrane, the *ex vivo* permeation study was carried out. The elastic liposomal gel (1 g) was evenly applied to the corneal membrane that separated the donor and receptor compartments. Simulated tear fluid was taken in the receptor region. The assembly was kept on the magnetic stirrer under constant stirring. The temperature was maintained at 37 °C. At pre-planned time intervals, the samples were taken. The amount of sample withdrawn was replenished with an equal amount of fresh media. UV spectrophotometer was used to evaluate the samples withdrawn at 261 nm. The percent cumulative drug permeated was determined using a calibration plot (Tiwari *et al*., 2020). The experiment was done in triplicate.

Data analysis (determination of flux and permeability coefficient): The amount of Fluconazole that permeated through the goat corneal membrane from the elastic liposome-based gel (Q, g/cm^2) was reported as a function of time (hr). The slope and intercept of the straight line generated by plotting the amount of fluconazole permeated against time under steady-state conditions were used to quantify the drug flux (permeation rate) at a steady state (Jss, g/cm^2/hr). The flux was divided by the initial drug concentration (Co) in the donor compartment of the cell to obtain the permeability coefficient (kp) (Tawfeek *et al*., 2020). The experiment was done in triplicate.

Determination of drug retention in the corneal membrane: Cotton soaked in a 0.05% sodium lauryl sulphate was used to remove the formulation that remained on the excised goat corneal membrane. Then the membrane was washed with distilled water. To extract fluconazole, the ocular membrane was weighed, chopped into small pieces, and sonicated for 15 minutes with methanol. After centrifuging and filtering the resultant solution, the drug concentration (g/cm^2) of the corneal membrane was measured using a UV spectrophotometer (Pathak *et al*., 2020). The experiment was done in triplicate.

Toxicity study: Toxicity studies were used to look into the formulation's safety. The following investigations were performed to see if the formulation had any unfavorable effects on the cornea of the eyes.

Corneal Hydration Test: The corneal hydration of goat corneas was determined using the same goat corneas used in the permeation study. Each cornea was weighed at the end of the experiment, then dipped in 1ml methanol, dried night long at 90°C, and was weighed again. The difference in weights was used to calculate corneal hydration (Zubairu *et al*., 2015). The experiment was done in triplicate.

Histopathological study: Each goat cornea was detached from the Franz diffusion cell and was kept in 10% formalin solution in distilled water after the *ex vivo* permeation study. Two untreated goat corneas were kept in potassium chloride solution and normal saline solution, respectively, before being fixed in 10% formalin solution. Following that, the slices were cut and stained with eosin and hematoxylin before being examined under a microscope (Zubairu *et al*., 2015).

Effect of formulation on corneocytes: To gain a better understanding of the tissue injury induced by formulation on corneocytes, a comparative toxicity study was performed. The assembly for the toxicity study was set using the same approach as the *ex vivo* permeation study assembly. In separate diffusion

cells, the drug-loaded formulation, normal saline, and a mixture of span 80 and soya lecithin were introduced into the donor compartment. Tyrode solution was poured into the receptor chamber. After 60 minutes, samples were obtained from the receptor chamber and analyzed utilizing the LDH assay kit from Coral clinical systems (Zubairu *et al*., 2015). The experiment was done in triplicate.

Bio adhesion testing: The experiment was carried out per the procedure reported in the literature by Zubairu et al. (21) with slight modifications. An agar plate was formulated. In the center of the plate, the test sample was placed. The prepared agar plate was attached to an IP disintegration test apparatus after 5 minutes after placing the test sample on it and was pushed up and down in simulated tear fluid at 37° C ± 1° C. At the lowest point, the sample on the plate was immersed in the solution, and at the highest point, it was out of the solution. The visual appearance of the formulation over the plate indicated the residence time of the elastic liposome-based gel on the plate (Zubairu *et al*., 2015). The experiment was done in triplicate.

Antifungal activity of the prepared gel by cup and plate method: The suspension of *Candida albicans* was prepared by taking a small quantity of the lyophilized powder of *Candida albicans* in a test tube containing 100 ml of nutrient broth and was incubated at 28° C for 24- 48 hours. After 24-48 hours, 50 µl of the suspension was added into 900 µl of sterile water. 50 µl of the suspension, which was prepared in sterile water, was grabbed and spread aseptically on Sabouraud dextrose agar plates using a sterile cotton swab, rotating the plates through a 60° C angle after each application. Finally, the swab was pushed along the agar surface's margins. With the lid closed, the plates were left to rest at room temperature. Then, using a sterile cork borer, three wells were bored into the agar medium and filled with elastic liposome-based gel (1g containing 5mg fluconazole), marketed gel (1g containing 5 mg fluconazole) and placebo gel (1g), respectively. To ensure equal drug distribution, the plates were placed in the refrigerator for two hours. The plates were incubated for 24-48 hours at 28° C. Around the wells; assessments were done for zones of inhibition. The inhibitory zones produced from all of the formulations evaluated were compared and results were reported (Kumar *et al*., 2012). The experiment was done in triplicate.

Stability studies: The gel formulation was stored at two different temperatures, at 4° C and room temperature for three months. The formulation's physical stability was then determined by visual inspection for phase separation. Additionally, pH, viscosity and drug content of the gel formulations were also evaluated (Shakeel *et al*., 2008).

Statistical analysis: Results were expressed as mean ± standard deviation (SD). The data obtained from various groups were statistically analysed using Graph Pad Instat 3, using two- tailed unpaired t-tests. Values at $p \leq 0.05$ were considered significant.

RESULTS

Preparation of elastic liposomes

Elastic liposomes were formulated using the rotary evaporation method. The optimised elastic liposomes exhibited particle size of 173.6 ± 5.9 nm, polydispersity index of 0.303 ± 0.03 and zeta potential of -10.0 ± (-0.311).

Preparation of elastic liposome-based gel

Selection of a gelling agent

To optimize the gelling agent, different gels were prepared using a 1% concentration of different gelling agents. Then the gels were characterized for various parameters. The results of the characterization of gels are given in Table 1 and based on the results of characterization carbopol 934 was selected for the development of elastic liposome- based gel.

Table 1: List of different gelling agents used for gel formulations

S. No.	Gelling agent	Concentration (w/v)	Observation	Homogeneity
1	Carbopol 934	1%	Very clear, stable, good consistency	Homogeneous
2	Carbopol 940	1%	Clear, hard	Non-homogeneous
3	Sodium alginate	1%	Phase separation	Non-homogeneous
4	Hydroxyl propyl methyl cellulose	1%	Grittiness and phase separation	Non-homogeneous

From the results, it was concluded that gels formulated using carbopol 934 were clear, stable and exhibited good consistency and therefore carbopol 934 was selected as the gelling agent. Further gels with different concentrations of carbopol 934 (from 0.5% to 2%w/v) were formulated.

Evaluation of elastic liposome-based gel

Physical evaluation: The different gels prepared were evaluated for physical appearance under normal daylight and the results are given in Table 2.

Table 2: Physical evaluation of different gel formulations

Property	E2 containing 0.5% carbopol 934	E2 containing 1% carbopol 934	E2 containing 1.5% carbopol 934	E2 containing 2% carbopol 934
Colour	Slightly yellowish	Slightly yellowish	Slightly yellowish	Slightly yellowish
Appearance	Opaque	Opaque	Opaque	Opaque
Odor	Characteristic	Characteristic	Characteristic	Characteristic
Washability	Washable	Washable	Washable	Washable
Consistency	+	++	+++	+++
Type of smear	Non greasy	Non greasy	Non greasy	Non greasy

(+) = low, (++) = medium, (+++) = good

pH: The pH of the different gel formulations prepared was determined and was found to be ranging from 7.0 to 7.3, indicating that the formulations prepared were safe for ocular administration. The results are given in Table 3.

Table 3: pH, drug content and viscosity of different gel formulations

S. No.	Gel formulations	pH± SD	Drug content % ± SD	Viscosity (mPa.s) ± SD
1	E2 (0.5% w/v)	7.1±0.01	88.3± 2.5	4361± 240
2	E2 (1% w/v)	7.3±0.02	95.1± 1.8	4984±250
3	E2 (1.5% w/v)	7.0±0.01	98.5±3.9	7217±340
4	E2 (2% w/v)	7.3±0.03	92.7±2.7	9969±410

Homogeneity and grittiness: Homogeneity and grittiness of different gel formulations prepared were determined and all the gel formulations were found to be homogeneous and free from gritty particles, indicating that the gel, when applied, would not cause any discomfort.

Drug content: The Drug content of the different gel formulations prepared was evaluated and was found to be ranging from 88.3 % to 98.5%. The results of the drug content determined are given in Table 3. Elastic liposome-based gel containing 1.5% w/v carbopol 934 exhibited a maximum drug content of 98.5%.

Rheological studies: The viscosity of the different gel formulations was determined using a rheometer (Anton- Paar). It was noticed that with the increase in the concentration of the gelling agent in a gel formulation, there was an increase in viscosity of the gel. The viscosity of the different gel formulations was found to

be ranging from 4361 to 9969 mPa.s. The results are given in Table 3.

In vitro release study: The *in vitro* release of the different gel formulations prepared was determined utilizing Franz diffusion cell and treated dialysis membrane. The percent cumulative drug release from the plain gel formulation was found to be significantly ($p \leq 0.05$) more (91±1.42 %) when compared with release from the elastic liposomal suspension gel over a period of six hours. The percent cumulative drug release from the different elastic liposomal gel formulations containing different concentration (0.5% w/v, 1% w/v, 1.5% w/v and 2% w/v) of gelling agent was found to be 86.45±0.107%, 85.4±0.18%, 80.5±0.32% and 77.7±0.21% respectively. From the results, it was noticed that with the rise in the concentration of the gelling agent, there was a significant decrease ($p \leq 0.05$) in the release of the drug from gel formulations. The elastic liposome-based gels showed continuous and sustained release of Fluconazole. *In vitro* release profile of different gel formulations and drug solution gel (plain gel) is given in Figure 1.

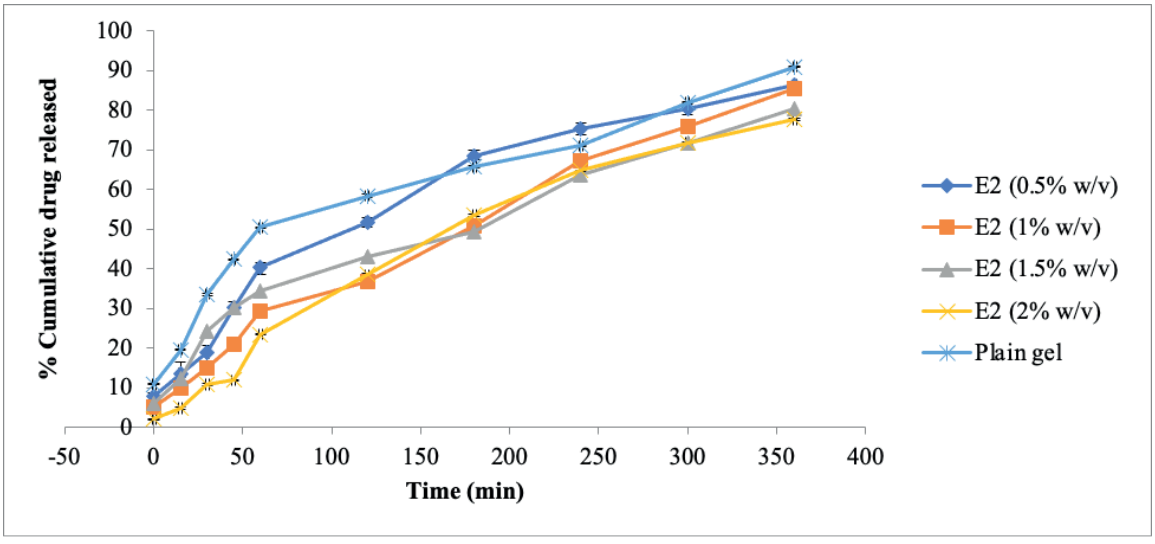

Figure 1: *In vitro* release profile of various gel formulations and drug solution gel (plain gel) (n=3)

For further studies, elastic liposome-based gel E2 $_{(1.5\%\ w/v)}$ was chosen instead of elastic liposome-based gel E2 $_{(2\%\ w/v)}$ because phase separation was observed in the case of elastic liposome-based gel E2 $_{(2\%\ w/v)}$ after 2 to 3 days.

Ex vivo permeation study: The *ex vivo* permeation study of the optimized gel formulation E2 $_{(1.5\%\ w/v)}$ was performed utilizing Franz diffusion cell and excised goat cornea for a period of six hours. The permeation of the optimized gel E2 $_{(1.5\%\ w/v)}$ was found to be significantly ($p \leq 0.05$) more (72.27±0.65 %) as compared to the plain gel (44.1±0.33 %), which could be attributed to the flexible and deformable nature of the elastic liposomes. Percentage cumulative amount of drug permeated versus time plot for optimized gel formulation E2 $_{(1.5\%\ w/v)}$ and plain gel formulation is given in Figure 2.

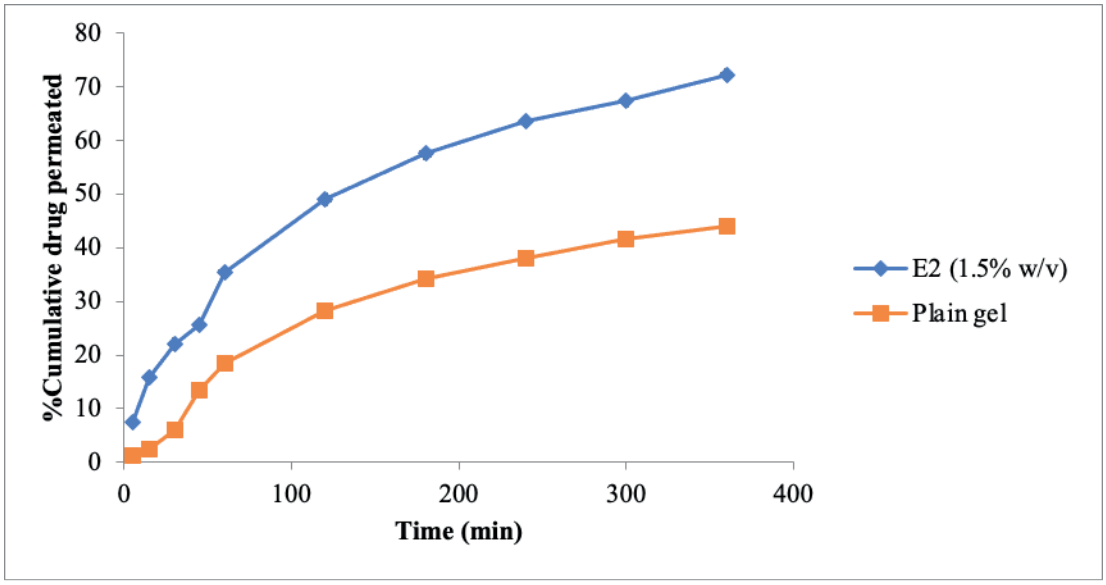

Figure 2: Percentage cumulative amount of drug permeated versus time plot for optimized gel formulation E2 $_{(1.5\%\ w/v)}$ and plain gel formulation (n=3)

Data analysis (determination of flux and permeability coefficient): Flux and permeability coefficient of the optimized gel formulation E2 $_{(1.5\%\ w/v)}$ were determined. The flux and permeability coefficient of E2 $_{(1.5\%\ w/v)}$ elastic liposomal based gel was significantly higher($p \leq 0.05$) as compared to plain gel formulation, which exhibited low flux and permeability coefficient. The results are shown in Table 4.

Table 4: Flux (J_{ss}) and permeability coefficient (k_p) of optimized gel formulation (E2 $_{(1.5\%\ w/v)}$) and plain gel formulation

S. No.	Formulations	Flux (µg/cm²/h) ± S.D (n=3)	Permeability coefficient (k_p)± S.D (n=3)
1	Optimized formulation (E2 $_{(1.5\%\ w/v)}$)	519.5± 0.110	0.1039 ± 0.002
2	Plain gel	367.8± 0.125	0.07356 ± 0.012

Determination of drug retention in the corneal membrane: Determination of drug retention (mg) and % drug retained in the corneal membrane was determined and significantly higher ($p \leq 0.05$) drug retention was observed in the case of optimized gel formulation E2 $_{(1.5\%\ w/v)}$. Drug retention (mg) and % drug retained in the case of optimized gel formulation E2 $_{(1.5\%\ w/v)}$ was found to be 1.05± 0.23 mg and 21±0.547 %, respectively. Drug retention and % drug retained in the case of plain gel were found to be 0.77± 0.11 mg and 15.4± 0.236 %, respectively.

Toxicity study

Corneal hydration test: Corneal hydration test was carried out and hydration was found to be 76.78%± 1.8%, which was within the range of (75- 80%) (Zubairu *et al.*) (Zubairu *et al.*, 2015), indicating that the formulation did not damage the cornea.

Histopathological study: Histopathological study was carried out and the examination revealed that the exposure of goat cornea to potassium chloride solution has shown considerable damage to the corneal tissues

(Figure 3), whereas no remarkable change was noticed in the corneal tissues treated with normal saline (Figure 4) and elastic liposomal based gel (Figure 5). The results indicated that elastic liposomal based gel did not cause any alteration in the structure of cornea thus conserving the histological structure of all corneal layers: the epithelium, the stroma and the endothelium. It was concluded that elastic liposome-based gel could be safely applied to the eye (Zubairu *et al*., 2015).

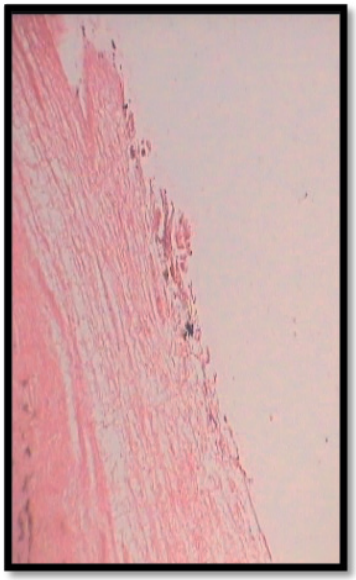

Figure 3: Goat cornea in saturated potassium chloride

Figure 4: Goat cornea in normal saline

Figure 5: Goat cornea treated optimized gel formulation

Effect of formulation on corneocytes: Biochemical estimation of LDH was carried out using a Coral clinical systems kit for LDH. LDH release in case of saline was found to be 11.9 ± 1.8 units/ cm^2, and LDH release in the case of elastic liposome-based gel was found to be 12.1 ± 1.3 units/ cm^2. In the case of span 80- soya lecithin mix, high LDH release was observed (18.62± 1.45 units/ cm^2) due to tissue destruction. Thus, a complete toxicological examination disclosed that the prepared elastic liposomal gel formulation had lower toxic potential.

After testing the levels of LDH in the Tyrode solution present in the receptor compartment, the goat corneas were homogenized using a tissue homogenizer. The homogenate obtained after homogenization was centrifuged. After centrifugation, the supernatant obtained was used for testing the LDH level. However, no significant difference was found in LDH levels in the case of normal saline (332.2± 1.2 units/ cm^2) and optimized elastic liposomal gel formulation (339.2 units/ cm^2), indicating that the optimized formulation was safe. On the contrary, span 80- soya lecithin mixture had a significantly higher (427.52 units/ cm^2) LDH level as compared to normal saline, further confirming that the elastic liposomal gel formulation ($E2_{1.5\%\ w/v}$) had lower toxic potential.

Bio adhesion testing: Bio adhesion testing of the optimized gel formulation was carried out, figure 6 shows the image of bio adhesion assembly. The bioadhesive potential of optimized elastic liposomal gel was compared to the marketed formulation (Flucos gel). From the results, it was observed that the optimized gel formulation ($E2_{(1.5\ \%\ w/v)}$) had a significantly ($p \leq 0.05$) higher (205± 5.5 min) bio adhesion time on the agar plate as compared to the marketed formulation (Flucos gel) (17± 0.5 min). The results indicated that the optimized gel formulation had better bio adhesion property than the marketed formulation.

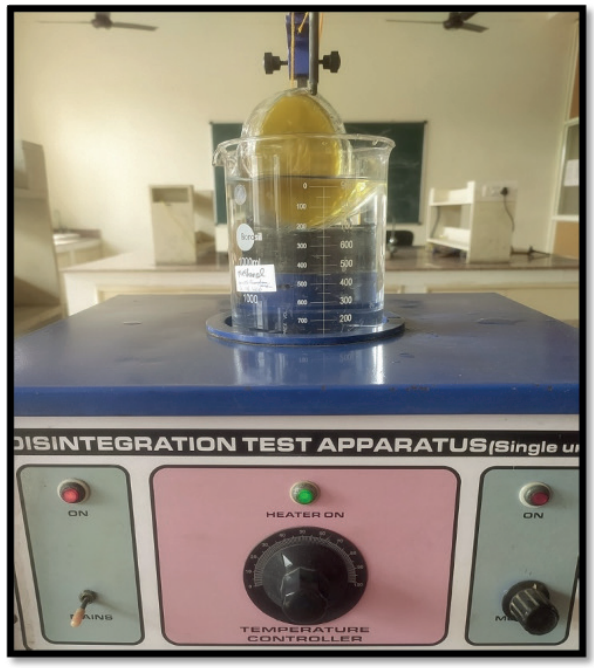

Figure 6: Bioadhesion testing assembly

Antifungal activity of the prepared gel by cup and plate method: Zone of inhibition for different formulations was determined. A significantly larger ($p \leq 0.05$) zone of inhibition was observed for elastic liposome-based gel E2 $_{(1.5\%\ w/v)}$ compared to the marketed formulation (Flucos gel) and placebo formulation, after 48 hours of the study period. The results are revealed in Table 5, Figure 7, and Figure 8.

Table 5: Zones of inhibition for Fluconazole loaded different formulations against *Candida albicans*

Formulations	Zone of inhibition (mm)	
	24 hrs± S.D (n=3)	**48 hrs± S.D (n=3)**
Elastic liposomal gel (El.G)	28 ± 0.9	34±1.4
Marketed gel (Flucos gel) (M.F)	18±1.1	20±1.0
Placebo gel (P.G)	0	0

Figure 7: Comparison of the zone of inhibition for various formulations evaluated against *Candida albicans* after incubation of 24 hours

(Here, El.G= Elastic liposome-based gel, P.G= Placebo gel and M.F= Marketed Formulation)

Figure 8: Comparison of the Zone of inhibition for various formulations evaluated against *Candida albicans* after incubation of 48 hours

Stability study: The stability study of elastic liposome-based gel E2 $_{(1.5\% w/v)}$ was carried out. The elastic liposome-based gel E2 $_{(1.5\% w/v)}$ (1g) was withdrawn at the end of 30, 60 and 90 days and was evaluated for pH, viscosity and drug content. The results are revealed in Table 6, Table 7, Figure 9 and Figure 10. No significant difference was observed in pH, viscosity, drug content and phase separation were not noticed in the elastic liposomal gel formulation, kept for stability study at 4° C.

Table 6: Stability study of optimized elastic liposome-based gel E2 $_{(1.5\% w/v)}$ when it was kept at 4° C

Time (Days)	pH ± S.D	Viscosity ± S.D	Drug content (mg) ± S.D	% Drug remaining	Log % drug remaining	Phase separation
0	7.0±0.036	7217±0.21	4.925± 0.023	100	2	No
30	7.01±0.041	7214±0.24	4.923± 0.025	99.96	1.9998	No
60	7.07±0.043	7213±0.29	4.919±0.029	99.87	1.9994	No
90	7.09±0.038	7211±0.25	4.915±0.031	99.8	1.9991	No

Figure 9: Gel formulation at the end of 90 days when it was kept at 4° C

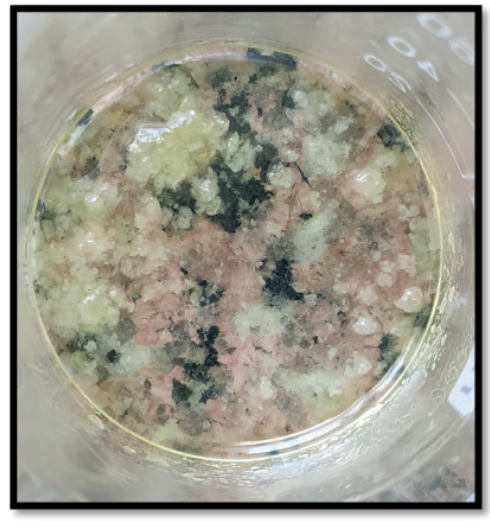

Figure 10: Gel formulation at the end of 30 days when it was kept at room temperature

Table 7: Stability study of optimized elastic liposome-based gel E2 $_{(1.5\%\ w/v)}$ when it was kept at room temperature

Time (month)	pH ± S.D	Viscosity ± S.D	Drug content (mg) ± S.D	% Drug remaining	Log % drug remaining	Phase separation
0	7.0±0.025	7217±0.19	4.925± 0.022	100	2	No

Phase separation was observed in elastic liposomal gel formulation after 30 days, when kept at room temperature.

So from the above results, it was concluded that the elastic liposomal gel formulation was more stable when kept at 4° C.

DISCUSSION

Preparation of elastic liposome-based gel

The optimized elastic liposomal suspension was incorporated into the gel to increase the retention time of the formulation in the eye. Enhanced retention would improve the therapeutic action by decreasing the lacrimal discharge of elastic liposomal suspension (Sayeh *et al*., 2014).

Evaluation of elastic liposome-based gel

In vitro release study: From the results obtained, it was observed that the elastic liposome-based gels showed continuous and sustained release of Fluconazole which may be because of the reservoir effect of elastic liposomes due to a combination of release from elastic liposomes first followed by diffusion through the gelling agent. The entrapped fluconazole molecules leaked out slowly from the vesicles into the enclosing gel and then into the release media, thereby providing a sustained effect. The advantage of sustained-release could be reduced application frequency and increased patient compliance (El-Gizawey *et al*., 2020).

Ex vivo permeation study: From the above *ex vivo* trans-corneal permeation studies, maximum permeation of Fluconazole was detected for the formulation E2 $_{(1.5\%\ w/v),}$ which was significantly higher ($p \leq 0.05$) than the plain gel formulation after 6-hour study. The higher permeation of Fluconazole from the elastic liposomal formulation E2 $_{(1.5\%\ w/v)}$ could be attributed to the presence of edge activators which render the formulation more flexible and permeable for transcorneal transport. The flux (519± 0.110 μg/cm^2/h) and permeability coefficient (0.1039) of E2 $_{(1.5\%\ w/v)}$ elastic liposomal based gel were significantly higher ($p \leq 0.05$) as compared to plain gel formulation which exhibited low flux (367.8± 0.125 μg/cm^2/h) and low permeability coefficient (0.07356).

The significantly higher ($p \leq 0.05$) permeation of Fluconazole from formulation E2 $_{(1.5\%\ w/v)}$ could be attributed to the smaller size of particles, the ability of elastic liposomes to squeeze through channels 1/10th of their diameter and their ability to penetrate deeper tissues (Benson, 2009). Another reason could be the presence of an edge activator, which worked as a permeation enhancer and played a significant role in enhancing the permeation (Tiwari *et al*., 2020).

Determination of drug retention in the corneal membrane: From the results obtained, a significantly higher amount of Fluconazole was localized into the cornea after application of elastic liposomal gel E2 $_{(1.5\%\ w/v)}$ as compared to plain gel formulation. More corneal retention in the case of elastic liposomal gel E2 $_{(1.5\%\ w/v)}$ was due to greater penetration of the drug from elastic liposomal gel due to nanosized elastic liposomal vesicles and their ability to penetrate through smaller pores and to penetrate deeper tissues (Benson, 2009). Besides this, another reason could be the establishment of an interaction with the outer structures of the eye, which results in the accumulation of elastic liposomes in the corneocytes. This interaction could allow systems to be released for more than 24 hours (Cristiano *et al*., 2019).

Toxicity study

Effect of formulation on corneocytes: In the case of span 80- soya lecithin mix, LDH release was observed to be significantly higher ($p \leq 0.05$) (18.62± 1.45 units/

cm^2), which could be attributed to tissue destruction. On the contrary, elastic liposomal gel formulation had a significantly lower (12.1± 1.3 units/ cm^2) LDH levels. As reported in the literature by Negi *et al.* (Negi *et al*., 2013), it may be due to the fact that surfactants bound to the vesicular system had a lower potential to cause cellular damage than free molecules.

Bioadhesion testing: The results indicated that the optimized gel formulation had better bioadhesion property than the marketed formulation. Due to better bioadhesion, the formulated elastic liposomal gel formulation would have better residence time on the eye, thereby improving the therapeutic efficacy with a reduction in dosing frequency.

Antifungal activity of the prepared gel by cup and plate method: A significantly larger zone of inhibition was observed for elastic liposome-based gel $E2_{(1.5\%\ w/v)}$ compared to marketed formulation (Flucos gel) and placebo formulation, after 48 hours of the study period. The larger zone of inhibition for elastic liposome-based gel $E2_{(1.5\%\ w/v)}$ could be attributed to nanosized particles of Fluconazole contained in elastic liposomes and due to the flexibility of elastic liposomes and ability to penetrate through small pores, which resulted in greater penetration through fungal cell walls, to inhibit ergosterol synthesis (Basha *et al*., 2013).

CONCLUSION

The elastic liposome gel was prepared successfully and was satisfactory in terms of release, bioadhesion and viscosity, with significantly higher penetration and better corneal drug retention. The present study endorsed that the elastic liposomal-based topical ophthalmic gel formulation was a good choice over conventional gel formulation for the treating keratomycosis.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

All the authors of this research declared no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Experimenting and the study text (RN), Developing hypothesis, Statistics, analysis and interpretation of the data (JKN), Literature Research (M), Histopathological experiments and interpretation of the data (RSN)

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RESEARCH ARTICLE

Evaluation of Anti-Inflammatory Activity of Metronidazole Treatment On Carrageenan Induced Paw Edema in Mice

Inci KAZKAYASI[*°], Gokcen TELLI[**]

Evaluation of Anti-Inflammatory Activity of Metronidazole Treatment On Carrageenan Induced Paw Edema in Mice

SUMMARY

Metronidazole is a nitroimidazole derivative antibiotic that has been used against protozoa and anaerobic organisms for a long time. Furthermore, it has been used in non-infectious inflammatory diseases such as acne, Crohn's disease, periorificial dermatitis, rosacea and seborrheic dermatitis recently. However, the studies about this issue are very few and its mechanism of action is unknown. The aim of our study is to evaluate the possible anti-inflammatory activity of metronidazole in vivo by using the mice- carrageenan-induced paw edema method. Mice were administered a single dose of 2, 20 or 200 mg/kg metronidazole via oral gavage. One hour later, 2% carrageenan was injected sub-plantar to the hind paws. The paw thickness of mice was measured just before the carrageenan injection and at 1, 2, 3, 4, 24 and 48 hours after injection by dial thickness gauge. For comparison, another group of mice received indomethacin (10 mg/kg, orally) used as a reference drug. IL-1β and TNF-α levels in the paws of mice were measured by the ELISA method. ANOVA (post-hoc Bonferroni) and Student's t tests were used for statistical analysis. Metronidazole displayed equi-potent anti-inflammatory activity with indomethacin in the carrageenan-induced mouse paw edema model. It is shown that less edema occurred at all doses (2, 20 and 200 mg/kg) compared to the control group and no differences were obtained in effect between the doses. It was observed that in metronidazole treated groups, paw thickness returned to baseline values 48 hours after carrageenan injection, unlike the control group. IL-1β and TNF-α levels, which were increased with carrageenan injection, were significantly decreased with metronidazole treatment. In our study, metronidazole was found to be anti-inflammatory due to its effects on relieving edema and reducing pro-inflammatory cytokines in the paws of carrageenan-induced mice. The effectiveness of metronidazole in treating various non-infectious diseases in recent years may be due to its anti-inflammatory activity.

Key Words: *Metronidazole, anti-inflammatory activity, mouse, paw-edema test*

Farelerde Karragenanla İndüklenmiş Pençe Ödem Modelinde Metronidazol Tedavisinin Anti-inflamatuvar Aktivitesinin Değerlendirilmesi

ÖZ

Metronidazol uzun yıllardır protozoalara ve anaerob bakterilere karşı kullanılan nitroimidazol türevi bir antibiyotiktir. Son yıllarda bunlara ek olarak, akne, Crohn hastalığı, periorifisyal dermatit, rozase ve seboreik dermatit gibi non-enfeksiyöz inflamatuvar hastalıklarda da kullanılmaya başlanmıştır. Ancak bu konudaki çalışmalar sınırlıdır ve bu etkinin mekanizması bilinmemektedir. Çalışmamızın amacı metronidazol'ün olası anti-inflamatuvar etkinliğinin karragenan ile indüklenen pençe ödemi yöntemi kullanılarak farelerde in vivo olarak gösterilmesidir. Deneylerde farelere gavaj yoluyla 2, 20 ya da 200 mg/kg metronidazol tek doz olarak uygulanmıştır. Bir saat sonra farelerin arka pençelerine inflamasyon oluşturmak için sub-plantar %2 karragenan enjeksiyonu yapılmış ve pençe kalınlığı karragenan enjeksiyonunun hemen öncesinde ve enjeksiyondan 1, 2, 3, 4, 24 ve 48 saat sonra mikrometrik kompas ile ölçülmüştür. Metronidazol'ün etkinliği, ayrı bir grup fareye referans ilaç olarak verilen (10 mg/kg, oral) indometazin ile karşılaştırılmıştır. Farelerin pençelerinde IL-1β ve TNF-α düzeyleri ELISA yöntemiyle ölçülmüştür. İstatistiksel analiz varyans analizi (post-hoc Bonferroni) ve Student's t test ile yapılmıştır. Farelerde karragenanla indüklenen pençe ödemi modelinde metronidazol, indometazinle benzer derecede anti-inflamatuvar etkinlik göstermiştir. Metronidazol her üç dozda (2, 20 ve 200 mg/kg) da kontrol grubuna kıyasla ödem oluşmasını engellemiştir ve etkide dozlar arasında anlamlı bir farklılık bulunmamıştır. Kontrol grubunda 48. saatte hala bir miktar devam eden ödem, metronidazol gruplarında kaybolmuştur. Karragenan enjeksiyonu ile artmış olan IL-1β ve TNF-α düzeyleri metronidazol tedavisi ile anlamlı olarak azalmıştır. Çalışmamızda metronidazol farelerde karragenan ile indüklenen pençe ödemini azaltıcı ve pro-inflamatuvar sitokinleri düşürücü etkileri nedeniyle anti-inflamatuvar bulunmuştur. Metronidazol'ün son yıllarda çeşitli non-enfeksiyöz hastalıkların tedavisindeki etkinliği anti-inflamatuvar aktivitesinden ileri gelebilir.

Anahtar Kelimeler: *Metronidazol, anti-inflamatuvar aktivite, fare, pençe-ödem testi*

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INTRODUCTION

Metronidazole is a nitroimidazole derivate antibiotic drug that has been used against protozoa and anaerobic bacteria for many years. It has been used alone or in combination with other antibiotics in many diseases such as pelvic inflammatory diseases, endocarditis and bacterial vaginosis. Besides its conventional use, it has been started to use both topically and orally in different indications such as acne vulgaris, Crohn's disease, periorificial dermatitis, rosacea and seborrheic dermatitis (Boeck, Abeck, Werfel, & Ring, 1997; Nishimuta & Ito, 2003; Pradhan, Madke, Kabra, & Singh, 2016; Seckin, Gurbuz, & Akin, 2007; Zip, 2010). These diseases are found to be related to inflammation (Dainichi, Hanakawa, & Kabashima, 2014) and it has been suggested that metronidazole has anti-inflammatory, immunosuppressive and antipruritic properties as well as antibacterial activity (Pradhan et al., 2016). Although the usage of metronidazole has become widespread, it is not known whether its effectiveness in such a wide range of diseases is due to its antibiotic, immunosuppressive or anti-inflammatory properties (Nishimuta & Ito, 2003).

There are a few studies in the literature investigating the anti-inflammatory effect of metronidazole. In one of them, metronidazole was shown to inhibit leukocyte-endothelial cell adhesion (Arndt, Palitzsch, Grisham, & Granger, 1994). In other studies, metronidazole altered the neutrophil activity and reduced the radical oxygen derivatives (Akamatsu et al., 1990; Del Rosso & Baum, 2008; Miyachi, 2001; Miyachi, Imamura, & Niwa, 1986). However, these studies are not sufficient for discussing the in vivo anti-inflammatory effects of metronidazole. Furthermore, the functional role of metronidazole on acute inflammation is not known yet. Therefore, in our study we aimed to induce inflammation acutely in mice by using the carrageenan to investigate the acute and long-time anti-inflammatory effect of metronidazole at increasing doses. Furthermore, we also investigated the possible mechanism of anti-inflammatory action of metronidazole, measuring some important cytokine levels in inflammation.

MATERIAL AND METHODS

Animals

Male, 8-12 weeks old Swiss albino mice were used in the experiments. Mice were housed in a room with a 12-hour day/night cycle at constant humidity and temperature (22 °C) and fed with a standard pellet diet. All animal use was approved by the Kobay DHL A.Ş. Local Ethics Committee (Decision approval date and number: 11.09.2020 and 503). All the procedures with animals were performed according to the rules of the "Guide for the Care and Use of Laboratory Animals".

Metronidazole Treatment

Metronidazole (Flagyl® 125 mg / 5 mL Oral Suspension, Sanofi Sağlık Ürünleri Ltd. Şti., İstanbul) was diluted to 0.3 mL of distilled water and administered to mice by gavage. The groups were administrated with 2, 20 or 200 mg/kg metronidazole as a single dose. Indomethacin (I7378, Merck, Darmstadt, Germany), known to be a well-established anti-inflammatory drug, was used as a reference drug (orally;10 mg/kg). The same volume of water was given to the control group mice by gavage. In each of the groups, eight mice were used.

2, 20 and 200 mg/kg doses of metronidazole were chosen in accordance with the dose previously applied to rats and took into consideration of the therapeutic dose for patients (Ganrot-Norlin, Stalhandske, & Karlstrom, 1981; Reagan-Shaw, Nihal, & Ahmad, 2008). Metronidazole, indomethacin, and distilled water were administered to mice 1 hour before carrageenan injection according to the study showed that the metronidazole reaches the maximum concentration in the blood one hour after oral administration (Ralph, 1983).

Carrageenan-induced Paw Edema Test

In vivo anti-inflammatory activity was evaluated

by a carrageenan-induced paw edema test (Posadas et al., 2004). Inflammation was induced by sub-plantar administration of 0.01 mL of 2% carrageenan (Sigma-Aldrich, Germany) into the hind paws of mice. The increase in paw thickness indicates inflammation-related edema and the reduction in edema reflects the anti-inflammatory activity of the administered drug. A dial thickness gauge was used to measure the paw thickness of mice (Dial Thickness Gauge, Ozaki Co., Japan; 0.01-1.0 mm). The paw measurements were performed just before (x_0) and 1, 2, 3, 4, 24 and 48 hours after the carrageenan injection (x) (Murat Ulu, 2019). The calculations were performed according to the formulas given below.

Change in paw thickness (Δ)=x- x_0

Edema inhibition (%) = [(Control Δ – Treatment Δ) / Control Δ] x 100

Potency was calculated according to the formula below. Because edema occurred mostly 2 hours after the carrageenan injection, the edema inhibition % of the 2nd hour was selected for the potency calculations.

Potency = [% edema inhibition of metronidazole of each animal (at 2nd hour) / the mean of % edema inhibition of indomethacin (at 2nd hour)] x 100

ELISA Assays

One series of mice were sacrificed at the second hour of injection. Their paws were isolated and froze immediately with liquid nitrogen. Tissues were kept at -80 °C until experiment day. Interleukin-1β (IL-1β) and Tumor Necrosis Factor alpha (TNF-α) levels were measured using ELISA kits (Invitrogen IL-1 beta Mouse ELISA Kit #BMS6002 and TNF alpha Mouse ELISA Kit #BMS607HS) according to the manufacturer's instructions.

Statistical analysis

Two-way repeated or one-way ANOVA and *post hoc* Bonferroni test was performed for statistical analysis. GraphPad Prism 5 Software (San Diego, USA) was used for analysis. Results are given as mean ± standard error of mean and a *p* value less than 0.05 is considered statistically significant.

RESULTS

Carrageenan-induced paw edema in the groups administered with 2, 20, and 200 mg/kg metronidazole was reduced at all measurement time points compared to the control group (Figure 1.).

Edema reached its highest level in the second hour in the control group and remained high until the fourth hour.

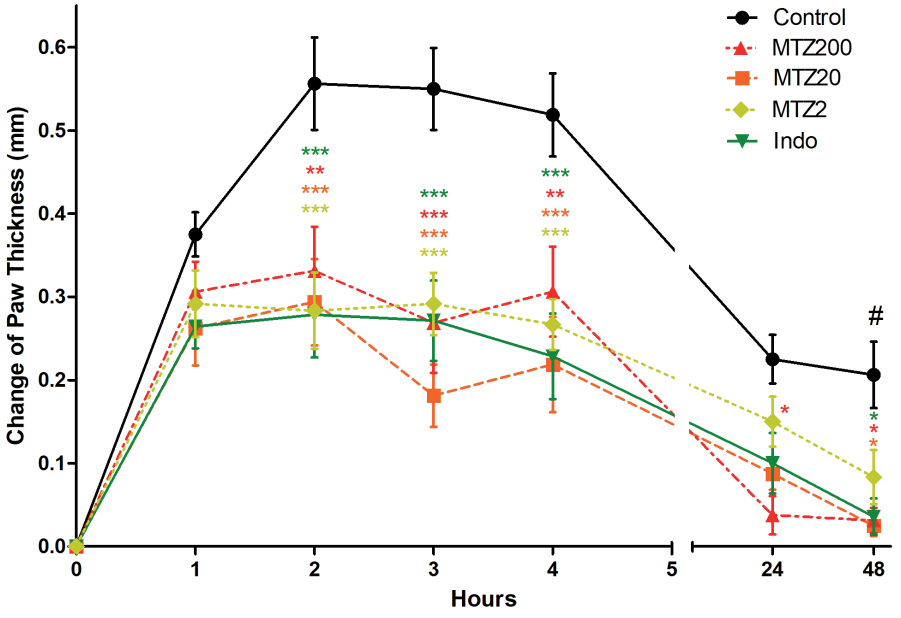

Figure 1. Changes of paw thickness after carrageenan injection

Figure 1. Time-dependent changes of paw thickness after carrageenan injection in mice treated with metronidazole (MTZ) (2, 20 and 200 mg/kg, orally). Data are expressed as mean ± standard error of mean. Eight mice were used for each group. Statistical analysis was performed with GraphPad Prism 5 Software. Two-way ANOVA on repeated measures and post-hoc Bonferroni test was used (* $p<0.05$; ** $p<0.01$; *** $p<0.001$ compared to time matched control group; #, $p< 0.001$ compared to initial paw thickness of control group).

One hour after carrageenan injection, the decrease of paw edema in metronidazole-treated mice did not show a statistically significant difference compared to the control group (Figure 1.). However, in the following hours, metronidazole significantly reduced carrageenan-induced paw edema at all doses compared to the control group (Figure 1.). There is no significant difference in edema relieving effect of metronidazole between different doses.

The developed edema did not dissipate even after 48 hours (#, $p=0.0019$) in the control group, whereas the paw thickness returned to the baseline values after 48 hours in all metronidazole groups (Figure 1.). Indomethacin significantly reduced carrageenan-induced paw edema compared to the control group (Figure 1.). There was no statistically significant difference between the metronidazole groups and the indomethacin group (Figure 1.). Metronidazole groups were found to be as potent as the indomethacin group, comparing the edema inhibition % of the metronidazole groups to the indomethacin group (Table 1.).

Table 1. Edema inhibition % and potency of metronidazole (MTZ) groups

	Indomethacin	MTZ 2 mg/kg	MTZ 20 mg/kg	MTZ 200 mg/kg
% Edema inhibition[a]	49,92 ± 9,23	49,06 ± 11,54	47,19 ± 9,32	40,45 ± 9,53
Potency[a]	100 ± 18,50	98,28 ± 23,12	94,53 ± 18,68	81,02 ± 19,08

[a]Comparison of edema inhibition % and potency of metronidazole (MTZ) groups with indomethacin group 2 hours after carrageenan injection. Data are given as mean ± standard error of mean.

The carrageenan injection in the paws of mice induced an inflammatory reaction in mice as shown by significantly increased levels of IL-1β and TNF-α (Figure 2.). Indomethacin decreased IL-1β and TNF-α levels compared to the control group. Metronidazole treatments at all doses also significantly reduced the levels of IL-1β and TNF-α compared to the control group (Figure 2.).

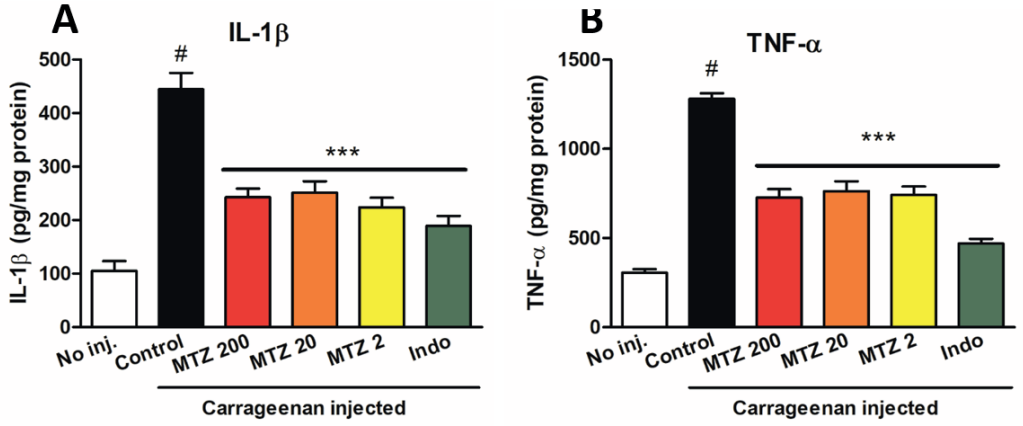

Figure 2. The effects of carrageenan injection on pro-inflammatory cytokines

Figure 2. The changes of pro-inflammatory cytokines after carrageenan injection in the paws of mice treated with metronidazole (MTZ) (2, 20 and 200 mg/kg, orally) or indomethacin (Indo) (10 mg/kg, orally). Data are expressed as mean ± standard error of mean. Four mice were used for each group. Statistical analysis was performed with GraphPad Prism 5 Software. One-way ANOVA and post-hoc Bonferroni test were used (# $p<0.001$ compared to the non-injected group; *** $p<0.001$ compared to control group).

DISCUSSION

Metronidazole is an antibiotic that has been prescribed for many years for its antibacterial and antiprotozoal properties. The action mechanism of metronidazole against anaerobic bacteria and protozoa is microbial DNA damage and nucleic acid synthesis inhibition (Edwards, 1993; Pradhan et al., 2016; Seidler Stangova et al., 2019). It has been used alone or in combination with other antibiotics in many diseases such as amebiasis, trichomoniasis, giardiasis, vaginitis, endocarditis, surgical chemoprophylaxis or *Helicobacter pylori* eradication (Chey, Leontiadis, Howden, & Moss, 2017; Petrin, Delgaty, Bhatt, & Garber, 1998). Since metronidazole is included in the first-line treatment known as triple therapy with proton pump inhibitor and clarithromycin for *Helicobacter pylori* infection, it is prescribed frequently (Chey et al., 2017).

Metronidazole has also been used in various non-infectious diseases in recent years (Khodaeiani et al., 2012; Pradhan et al., 2016; Seidler Stangova et al., 2019; Zip, 2010). Topical gels or creams are effective in rosacea, acne vulgaris, periorificial dermatitis and seborrheic dermatitis (Boeck et al., 1997; Del Rosso & Baum, 2008; Khodaeiani et al., 2012; Nishimuta & Ito, 2003; Seckin et al., 2007; Zip, 2010). It has been suggested that the therapeutic effect of metronidazole in rosacea and acne vulgaris may be due to its anti-inflammatory effect rather than its antibacterial effect (Nishimuta & Ito, 2003). However, the action mechanism of metronidazole in the treatment of various non-infectious diseases is not fully understood. In one of the previous studies about its anti-inflammatory activity, metronidazole prevented leukocyte endothelial adhesion (Arndt et

al., 1994). In other studies, it has been suggested that metronidazole may have an anti-inflammatory effect by changing neutrophil activity and reducing radical oxygen derivatives (Akamatsu et al., 1990; Del Rosso & Baum, 2008; Miyachi, 2001; Miyachi et al., 1986).

In our study, the anti-inflammatory activity of metronidazole was investigated in a carrageenan-induced paw edema model in mice. Metronidazole showed significant anti-inflammatory activity at all three doses via reducing carrageenan-induced edema and two important pro-inflammatory cytokines. We selected three doses of metronidazole for scanning a large dose-effect range and assessing the dose-response relation of metronidazole and its anti-inflammatory activity of it. The 20 mg/kg dose was chosen in accordance with the dose of metronidazole used in various infectious diseases in the clinic. When the body surface area normalization method was used instead of a simple conversion based on body weight for dose translation (Reagan-Shaw et al., 2008), the dose of metronidazole was calculated as 200 mg/kg for mice. Thus, a group of mice administered 200 mg/kg was also created. Finally, the low (2 mg/kg) dose was administered to test the subclinical effect. We found no difference in preventing edema between the groups that received increasing doses of metronidazole, suggesting that the anti-inflammatory effect was not dose-dependent in this dose range. According to our findings, it can be suggested that metronidazole may have an anti-inflammatory effect besides its antibacterial effect at the dose used in the treatment of various bacterial diseases in the clinic (e.g. 500 mg or 3x250 mg).

Acute inflammation is characterized by edema that develops as a result of increased vascular permeability, extravasation of fluids and proteins, cellular infiltration and accumulation of leukocytes in the inflammation area (Necas & Bartosikova, 2013; Posadas et al., 2004). The carrageenan-induced paw edema model is a well-known and widely used acute inflammation model (Necas & Bartosikova, 2013; Xue, Wu, Wu, Li, & Wang, 2019). Carrageenan injection develops edema, one of the five cardinal signs of inflammation, without causing any injury or damage to the inflamed paw (Necas & Bartosikova, 2013; Telli, Kazkayasi, & Uma, 2021). Indomethacin is a non-steroidal anti-inflammatory drug with strong anti-inflammatory activity. It has been used as a reference drug in the carrageenan-induced paw edema model (Akindele & Adeyemi, 2007). Therefore, indomethacin was used as a reference drug in our study and metronidazole groups were found to be as potent as it is.

In the carrageenan-induced paw edema model, it was observed that the control group developed severe edema starting from the second hour of injection, which is consistent with previous studies (Necas & Bartosikova, 2013; Posadas et al., 2004). In our study, measurements were continued until the 48th hour. The paw thickness of control group did not return to the initial values even 48 hours after injection. On the other hand, the paw thickness of metronidazole groups at 48th hour was not different from the initial value, indicating that the anti-inflammatory effect persisted.

IL-1β, which stimulates the local and systemic inflammation responses of the body is a prototypical cytokine. It stimulates the accumulation of inflammatory cells by inducing the release of adhesion molecules on endothelial cells. It also triggers the synthesis of various enzymes, which contribute to the release of inflammatory mediators (Gabay, Lamacchia, & Palmer, 2010). In our study, carrageenan injection increased IL-1β level which was decreased by metronidazole treatments. Another pro-inflammatory cytokine TNF-α, regulates vascular endothelium and endothelial leukocyte interactions. This interaction in combination with the release of chemokines may lead to the recruitment of different populations of leukocytes. In addition, TNF-α contributes to the inflammation process by inducing fever, vasodilatation and expression of pro-coagulant

proteins (Bradley, 2008). Similarly, to IL-1β, we found that carrageenan injection increased TNF-α level which was decreased by metronidazole treatments. In accordance with our results, it has been shown that metronidazole incubation was shown to reduce IL-1β and TNF-α levels in lipopolysaccharide-induced human periodontal ligament cells (Rizzo et al., 2010). Thus, the mechanism underlying the anti-inflammatory effect of metronidazole may be due to its IL-1β and TNF-α reducing effect.

CONCLUSION

In conclusion, our study showed for the first time that metronidazole has anti-inflammatory activity in acute inflammation *in vivo* by using a carrageenan-induced paw edema model in mice. Furthermore, it has been shown that pro-inflammatory cytokines, IL-1β and TNF-α, which were increased with carrageenan injection, decreased with metronidazole treatment. If its anti-inflammatory activity can be demonstrated in different inflammation models with further studies, the rationale for using metronidazole in various non-infectious diseases may be attributed to its anti-inflammatory activity and metronidazole can be used in the treatment of many other inflammatory diseases.

CONFLICT OF INTEREST

The authors of this article declared no conflict of interest.

AUTHOR CONTRIBUTIONS

IK developed the hypothesis, did the literature research, experiments and data analysis and wrote the paper. GT did the experiments, reviewed and edited the paper.

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RESEARCH ARTICLE

Studying the Protective Effect of Ellagic Acid Against High Glucose-Associated Toxicity in H9C2 Cardiomyocytes

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Studying the Protective Effect of Ellagic Acid Against High Glucose-Associated Toxicity in H9C2 Cardiomyocytes

SUMMARY

Diabetes mellitus leads to an increased risk factor for cardiovascular diseases. Accumulating evidence has demonstrated that high glucose (HG) can promote massive apoptosis in cardiomyocytes. Oxidative stress has been known as main factor responsible for HG-induced apoptosis. Ellagic acid, a natural phenolic compound, exhibits anti-inflammatory, anti-atherogenic, and antioxidant effects. This study was carried out to evaluate the effects of ellagic acid on HG-induced oxidative damage in H9C2 cells. The effect of ellagic acid on the viability of cells was evaluated by the MTT method. The oxidative stress parameters, including levels of malondialdehyde (MDA), glutathione (GSH), total antioxidant capacity (TAC), and superoxide dismutase (SOD) activity were also measured. The results indicated that ellagic acid (10 μM and 20 μM) could remarkably enhance the cell viability of H9C2 cells exposed to HG. In addition, ellagic acid significantly improved the levels of intracellular GSH, TAC, and SOD, whereas the levels of MDA were attenuated. These results revealed a protective effect of ellagic acid on HG-induced cytotoxicity, at least partially, by increasing antioxidant activity and preventing oxidative stress.

Key Words: *H9C2 cells, high glucose, ellagic acid, cardiotoxicity, oxidative stress*

H9C2 Kardiyomiyositlerde Yüksek Glikoz Bağlantılı Toksisiteye Karşı Elajik Asitin Koruyucu Etkisinin İncelenmesi

ÖZ

Diabetes mellitus, kardiyovasküler hastalıklar için riskin artmasına neden olan bir faktördür. Elde edilen veriler, yüksek glikozun (HG) kardiyomiyositlerde yaygın apoptozu teşvik edebileceğini göstermiştir. Oksidatif stres, HG'nin neden olduğu apoptozdan sorumlu ana faktör olarak bilinmektedir. Doğal bir fenolik bileşik olan elajik asit, anti-inflamatuar, anti-aterojenik ve anti-oksidan etkiler göstermektedir. Bu çalışma, elajik asidin H9C2 hücrelerinde HG'nin neden olduğu oksidatif hasar üzerindeki etkilerini değerlendirmek için yapılmıştır. Elajik asitin hücre canlılığı üzerindeki etkisi MTT yöntemi ile değerlendirilmiştir. Malondialdehit (MDA), glutatyon (GSH), toplam antioksidan kapasite (TAC) ve süperoksit dismutaz (SOD) aktivitesini içeren oksidatif stres parametreleri de ölçülmüştür. Sonuçlar, elajik asitin (10 uM ve 20 uM), HG'ye maruz kalan H9C2 hücrelerinin hücre canlılığını önemli ölçüde artırabildiğini göstermiştir. Ek olarak, elajik asit, hücre içi GSH, TAC ve SOD düzeylerini önemli ölçüde artırırken, MDA düzeylerini azaltmıştır. Birlikte ele alındığında, bu sonuçlar, elajik asitin, en azından kısmen, antioksidan aktiviteyi artırarak ve oksidatif stresi önleyerek, HG'nin neden olduğu sitotoksisite üzerinde koruyucu bir etkisini ortaya koymuştur.

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INTRODUCTION

Diabetes mellitus (DM) is a leading cause of many chronic conditions, including nephropathy, retinopathy, neuropathy, and cardiovascular diseases (Lin, 2014; Chawla, 2016). High glucose (HG), the most important feature of DM, can cause the development of cardiovascular diseases (Martín-Timón, 2014). It has been shown that HG stimulates myocardial apoptosis through oxidative stress and inflammatory reactions (Yan, 2011). It has been recently indicated that HG leads to the overproduction of reactive oxygen species (ROS), ultimately resulting in the activation of the apoptotic pathway (Kumar, 2012). ROS are chemically reactive chemical species containing oxygen. Oxidative stress is a condition characterized by increased intracellular levels of ROS. It is known that oxidative stress has an essential role in the pathogenesis of cardiovascular complications of diabetes (Haidara, 2006). Oxidative stress can result in the reduction of antioxidant defense proteins such as glutathione (Cnubben, 2001). Furthermore, free radicals cause modifications in proinflammatory mediators such as nuclear factor kappa B (NF-κB) and suppress nuclear factor erythroid 2-related factor 2 (Nrf2), which are major transcription factors implicated in antioxidant capacity (Videla, 2009). These events ultimately lead to ROS attack on proteins, lipid membranes, and DNA, causing cardiac cell damage and apoptosis (Datta, 2000). Therefore, natural antioxidant scavengers such as polyphenol compounds can be useful in reducing the free radicals in cardiac cells. Studies have shown that polyphenol compounds have beneficial effects on cardiovascular diseases (Quiñones, 2013). Ellagic acid is a natural polyphenolic compound found in blackberry, strawberry, bayberry, pineapple, and pomegranate (Amakura, 2000). It is reported to have important biological properties including antimicrobial, anti-inflammatory, anti-atherogenic, and anti-mutagenic effects (Rogerio, 2006; Abuelsaad, 2013). Ellagic acid has been shown to have potent antioxidant activity that effectively scavenges ROS (Devipriya, 2007). Moreover, studies have shown that ellagic acid exhibits beneficial effects in the treatment of hypertension and prevention of nephrotoxicity (Al-Kharusi, 2013; Berkban, 2015). Studies have indicated that the antioxidant activity of polyphenols plays an important role against oxidative stress-induced injury in cardiomyocytes (Mattera, 2017). Trolox, a vitamin E analog, inhibits oxidative damage to H9C2 cells under HG condition (Davargaon, 2019). Interestingly, one study reported that ellagic acid provided more effective protection than vitamin E against oxidative stress damage in embryonic and placental tissues of C57BL/6J mice (Hassoun, 1997). H9C2 cells are proper *in vitro* model for cardiac research studies (Zordoky & El-Kadi, 2007). Therefore, the present study aimed to examine the protective effects of ellagic acid on HG-induced oxidative injury in H9C2 cells with a focus on its antioxidant properties.

MATERIALS AND METHODS

Cell culture

The H9C2 cells were purchased from the National Cell Bank of Iran (NCBI code: C585, Pasteur Institute of Iran, Tehran, Iran). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Invitrogen, Carlsbad, CA, USA) containing 4 mM L-glutamine, 1 mM sodium pyruvate, 1500 mg/L sodium bicarbonate, and 1000 mg/L glucose. The culture medium was supplemented with 1% penicillin-streptomycin (Gibco, USA) and 10% heat-inactivated fetal bovine serum (FBS, Gibco, USA). The cells were maintained in 5% CO2 and 37°C.

Determination of cell viability

The effect of ellagic acid on HG toxicity was measured via the analysis of viable cells using MTT colorimetric assay. Briefly, 1×10^4 H9C2 cells were seeded into each well of a 96-well plate. After 24 h incubation, the cells were treated with different glucose concentrations (20, 35, 50, and 65 mM) for 24 h. The level of 50 mM glucose was chosen for the next steps. Then, the cells were pretreated with various concentrations of ellagic acid (5, 10, 20, and 30 μM, Sigma Aldrich, St Louis, MO, USA) for 24 h and next, the cells were

treated with HG (50 mM) for 24 h. After the above mentioned time, 10 µM of MTT (5 mg/ml, Sigma, USA) was added to each well and the plates were incubated for 3-4 h at 37°C. Then, the medium was removed and 100 µM of DMSO was added to each well and the absorbance was measured with an ELISA plate reader at 570 nm.

Thiobarbituric acid reactive substances (TBARS) assay

Malondialdehyde (MDA), a three-carbon low molecular weight aldehyde, is one of the end products of lipid peroxidation (LPO) in the cells. MDA level has been implicated as a marker of oxidative stress and antioxidant status (Gaweł, 2004). After treatment, the H9C2 cell lines (3×10^6 cells) were washed twice with PBS and then cells were centrifuged at 3000 g for 5 min. Then, 200 µl of the sample was mixed with 500 µl of 20% trichloroacetic acid (Sigma, USA), 400 µl of thiobarbituric acid (TBA, Sigma, USA), and 500 µl of sulfuric acid (Merck Company, Darmstadt, Germany). Then it was heated for 30 min in a boiling water bath. After cooling, samples were mixed with 0.8 ml of n-butanol (Merck, Germany), next centrifuged at 4100 g for 12 min. The quantification of MDA levels in the samples was measured in the absorbance of 532 nm.

Measurement of total thiol groups

The total thiol groups were determined by the HU method (Hu, 1994). 5, 5'-dithiobis-(2-nitrobenzoic acid), also called DTNB or Ellman's reagent, is a versatile water-soluble compound for evaluating sulfhydryl groups in the solution. Briefly, the cell lysates were added to Ellman's reagent (5 mM, Sigma, USA), and centrifuged for 10 min. The absorbance of the mixture was measured at 412 nm.

Measurement of total antioxidant capacity (TAC) levels

The TAC levels were determined by the ferric reducing antioxidant power (FRAP) assay. This method was based on the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) in the presence of tripyridyltriazine (TPTZ) by the colorimetric method. Briefly, the FRAP reagent containing TPTZ (10 mM, Sigma, USA), HCl (40 mM, Merck, Germany), $FeCl_3$ (20 mM, Merck, Germany), and acetate buffer was freshly prepared and added to the samples. The mixture was incubated for 5 min at 37°C, and the absorbance at 593 nm was recorded using an ELISA plate reader.

Measurement of superoxide dismutase (SOD)

SOD is an essential antioxidant defense against oxidative stress in the body. SOD is the first line of physiological defense and most strong antioxidant in the cells (Tsai, 2015). To assay for SOD activity, 610 µl of PBS and 90 µl of pyrogallol (Sigma, USA) were added to the supernatant. The absorbance was measured at 420 nm (Marklund & Marklund, 1974).

Statistical methods

All data were represented as the mean ± S.D in GraphPad Prism 5.0 software (San Diego, CA, USA). Statistical analyses were evaluated using the one-way ANOVA followed by Tukey's post hoc test. A *P* value less than 0.05 was assumed to be statistically significant.

RESULTS

Effect of high glucose on cell viability

To identify the effect of HG on cell viability, MTT reduction assay was used. H9C2 cells were exposed to different glucose concentrations (20, 35, 50, and 65 mM) for 24 h. The results showed that HG (35, 50, and 65 mM) decreased cell viability in H9C2 cells in a concentration-dependent manner. HG in the concentration of 50 mM was selected for experiments ($P < 0.001$) (Figure 1).

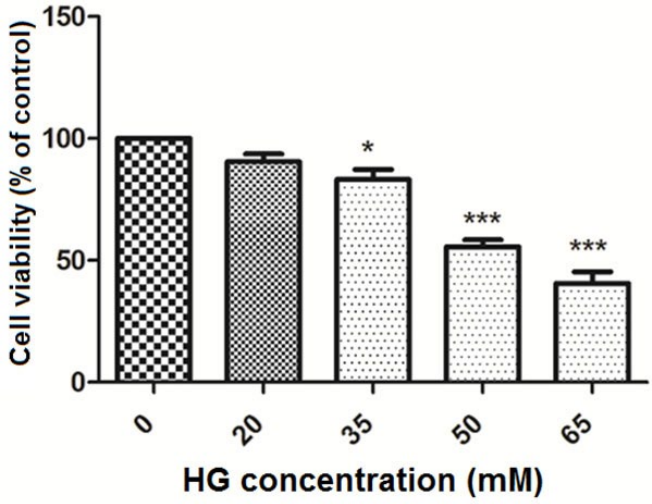

Figure 1. Cell viability of H9C2 cells after treatment with various concentrations of glucose. The values are expressed by mean ± S.D; n=5. $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with the control group.

Ellagic acid attenuated HG-induced loss of cell viability in H9C2 cells

The H9C2 cell lines were pre-incubated with ellagic acid followed by exposure to HG. The results showed that ellagic acid significantly increased cell viability as compared with the HG group. Because ellagic acid at concentrations of 10 μM and 20 μM produced significant protective effects, these concentrations were chosen for use in this study ($P < 0.001$ for both concentrations) (Figure 2).

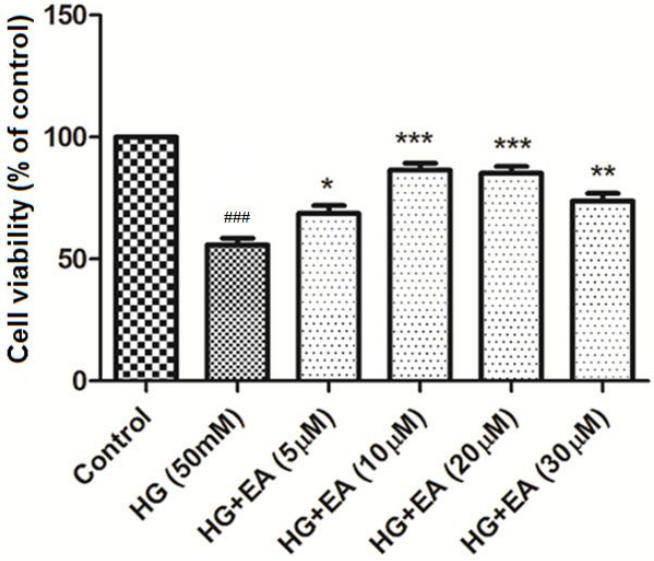

Figure 2. Effects of ellagic acid (EA) on cell viability in HG-treated H9C2 cells. The values are expressed by mean ± S.D; n=5. $^{###}P < 0.001$ compared with the control group; $^{*}P< 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were compared with the HG group.

Effect of ellagic acid on LPO in H9C2 cells

To evaluate whether ellagic acid is involved in HG-induced LPO, the cells were pre-incubated with ellagic acid and then treated with HG. Our results indicated that HG increased the level of MDA. Treatment of H9C2 cells with ellagic acid (10 μM and 20 μM) decreased MDA levels as compared with HG ($P < 0.001$ for both groups) (Figure 3a).

Ellagic acid restored HG-induced decrease in the activity of GSH

Our results showed that HG significantly reduced GSH levels compared with control ($P< 0.001$), whereas pretreatment with ellagic acid (10 μM and 20 μM) for 24 h, significantly increased the GSH levels compared with H9C2 cells exposed to HG ($P < 0.001$ for both groups) (Figure 3b).

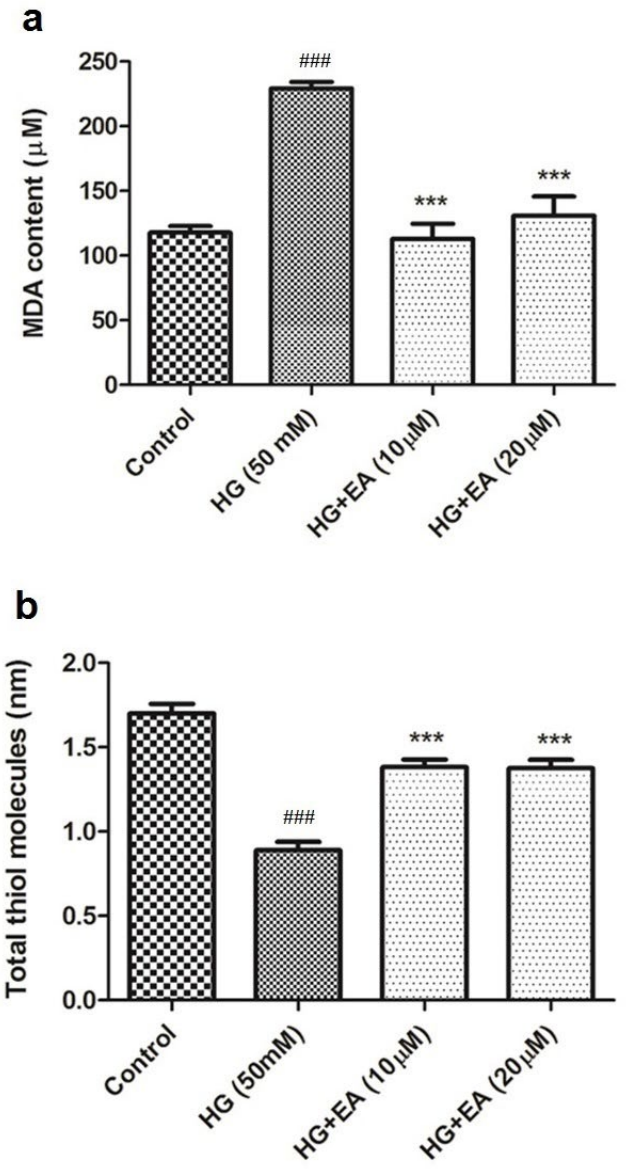

Figure 3. Effects of EA on MDA and GSH levels in HG-treated H9C2 cells. The values are expressed by mean ± S.D; n=3. $^{###}P < 0.001$ compared with the control group; $^{***}P < 0.001$ was compared with the HG group.

Ellagic acid attenuated HG-induced decrease in TAC levels

Effects of ellagic acid on TAC levels were studied by FRAP assay. As shown in Figure 4a, results illustrated that the HG condition markedly reduced TAC levels while ellagic acid (10 μM and 20 μM) significantly enhanced TAC levels ($P < 0.001$ and $P < 0.01$, respectively).

Ellagic acid increased HG-induced decrease in SOD activity

As shown in Figure 4b, SOD activity is markedly reduced in the HG-exposed group ($P < 0.001$). However, ellagic acid administration (10 μM and 20 μM) effectively increased SOD activity as compared with the HG group in H9C2 cells ($P < 0.001$ for both concentrations).

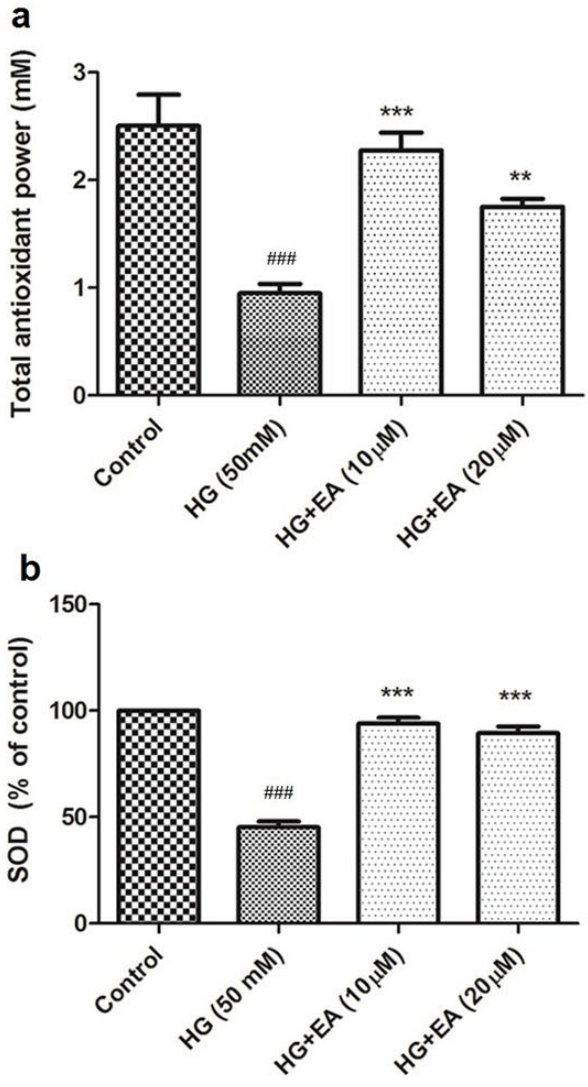

Figure 4. Effects of EA on total antioxidant capacity and SOD levels in HG-treated H9C2 cells. The values are expressed by mean ± S.D; n=3. $^{###}P < 0.001$ compared with the control group; $^{**}P < 0.01$ and $^{***}P < 0.001$ were compared with the HG group.

DISCUSSION

This study was designed to examine the cardioprotective effect of ellagic acid on HG-induced oxidative stress in H9C2 cardiac cells. We observed that ellagic acid prevented HG-induced cytotoxicity in H9C2 cells by decreasing the levels of MDA and increasing the levels of intracellular GSH, SOD, and TAC.

Clinical studies have shown that cardiovascular disease (CVD) is a major cause of death in patients with diabetes (Martín-Timón, 2014). It has been shown that oxidative stress has a crucial role in CVD development (Haidara, 2006). Previous studies demonstrated that HG stimulated apoptosis in vascular endothelial cells via the accumulation of ROS levels (Kumar, 2012; Aminzadeh & Bashiri, 2020). Furthermore, studies have shown that cardiac myocyte apoptosis under pathological conditions plays a major role in CVD. Experimental and clinical diabetic models have shown that oxidative stress mediated heart injury (Haidara, 2006). Excessive oxidative stress is an imbalance between ROS production and antioxidant defense systems, which plays an essential role in inducing apoptosis. Several biomarkers are used to evaluate oxidative stress conditions, such as levels of lipid peroxidation, SOD, catalase, and GSH.

Antioxidants organize the primary defense system that limits the toxicity associated with oxidative stress. Some studies have previously shown that antioxidant natural substances such as diallyl trisulfide could prevent HG-induced apoptosis in cardiac cells. It was shown that antioxidants could improve heart disease in diabetic conditions after the administration of streptozotocin (Kumar, 2013). It is also indicated that polyphenolic compounds have protective roles on different noxious stimuli, including rat model of Parkinson's disease and lipopolysaccharide-induced inflammation in RAW264 cells (Baluchnejadmojarad, 2017; Du, 2019; Wang, 2019).

In this research, we demonstrated that HG induced H9C2 cells injury while ellagic acid could prevent oxidative stress production in cardiac cells. H9C2 cells were pre-incubated with various concentrations of ellagic acid (5-30 μM) and the effect of ellagic acid on viability was determined by MTT assay. Our results showed that cell viability decreased when H9C2 cells were incubated with HG. Pre-incubation with ellagic acid successfully increased cell viability. This result is in agreement with previous findings indicating that ellagic acid enhanced cell viability and also provided cytoprotection against oxidized LDL-induced apoptosis in primary human umbilical vein endothelial cells (Ou, 2010).

Increasing documents in experimental and clinical studies propose that free radicals lead to increased lipid peroxidation and MDA content. The MDA level has been recognized as a circuitous parameter of oxidative stress in cells. Lipid peroxidation has been involved in the pathogenesis of various disorders such as diabetes, atherosclerosis, fibrosis, and cancer (Negre-Salvayre, 2010; Aminzadeh & Salarinejad, 2019). Lipid peroxidation can either directly degrade enzymes by free radicals, or by chemical changes in end products (Halliwell & Chirico, 1993). Our findings revealed that HG enhanced the levels of MDA in cardiac cells. A similar study reported that elevated level of MDA in diabetic model was induced by low dose of streptozoticin in rats (Yu, 2012). Moreover, this study showed that ellagic acid prevents the increased in MDA levels in HG condition. In line with current results, evidence indicates that ellagic acid inhibited LPO and attenuated the high-fat diet/low dose streptozotocin-induced diabetic nephropathy in rats (Ahad, 2014).

GSH is a common molecule found in many tissues and cells. GSH, a crucial regulator of intracellular oxidative balance, has an important role in cell death principally by binding to cysteine residues in proteins (Anderson, 1998). In the present study, our results showed that HG decreased GSH levels in H9C2 cells. We observed that pretreatment with ellagic acid increased GSH levels as compared with the HG group. Our findings are consistent with reports indicating that ellagic acid restored GSH levels on H_2O_2-induced apoptosis in PC12 cells (Pavlica & Gebhardt, 2005).

SOD is one of the most essential enzymes in the antioxidant defense system (Yim, 1990). In the present study, we observed that treatment of H9C2 cells with HG decreased SOD activity, while ellagic acid effectively increased SOD activity as compared with the HG group. The results of the present study are in agreement with studies indicating that ellagic acid enhances the SOD levels to protect against 2, 2-diphenyl-1-picrylhydrazyl (DPPH)-induced toxicity in V79-4 cells (Han, 2006).

CONCLUSION

Overall, the present study revealed a protective role of ellagic acid against HG-induced cardiotoxicity in H9C2 cells *in vitro.* Our results demonstrated that pretreatment of H9C2 cells with ellagic acid reduced the oxidative stress in HG condition. The antioxidant role of ellagic acid could be responsible for this activity.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Developing hypothesis, literature research, analysis and interpretation of the data, reviewing the text (Aminzadeh, A.), experimenting, preparing the study text, analysis and interpretation of the data (Keshavarzi, E.)

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RESEARCH ARTICLE

Digital Data Security Awareness: A Study with Pharmacy Students

Nilay TARHAN[*°]

Digital Data Security Awareness: A Study with Pharmacy Students

SUMMARY

Technological developments lead to changes in the field of health as well as in many areas. Although these developments bring many advantages, there are also some potential risks. Increments in digital healthcare and digitally stored information raise concerns about data security. It is vital for pharmacists and pharmacy students to be aware of data security, considering patient rights and ethical principles. This study investigates the digital data security awareness of pharmacy students and the effects of some variables on it. In this context, a questionnaire was applied to pharmacy students, including the "Digital Data Security Awareness Scale" developed by Yılmaz, Şahin, & Akbulut (2015). Firstly, exploratory factor analysis was performed on the data obtained, and then the effects of some variables on digital data security awareness were investigated via t-test and analysis of variance. Digital data security awareness is found to be higher in those who have an antivirus program on their smartphones. Additionally, it was determined that the mean of the responses given by the students to the statements on the scale was around three. As a result, the awareness of pharmacy students on this issue needs to be improved.

Key Words: *Data security, pharmacy practices, ethics*

Dijital Veri Güvenliği Farkındalığı: Eczacılık Öğrencileri ile Bir Çalışma

ÖZ

Teknolojik gelişmeler, sağlık alanında da birçok alanda olduğu gibi değişikliklere yol açmaktadır. Bu gelişmeler beraberinde birçok avantaj getirse de bazı potansiyel riskler de bulunmaktadır. Dijital sağlık hizmetlerindeki ve dijital olarak depolanan bilgilerdeki artış, veri güvenliği konusundaki endişeleri de arttırmaktadır. Eczacıların ve eczacılık öğrencilerinin, hasta hakları ve etik ilkeler de göz önünde bulundurulduğunda, veri güvenliği konusunda farkındalığa sahip olmaları oldukça önem arz etmektedir. Bu çalışma, eczacılık öğrencilerinin dijital veri güvenliği farkındalığını ve bazı değişkenlerin buna etkilerini incelemektedir. Bu kapsamda eczacılık öğrencilerine Yılmaz, Şahin, ve Akbulut (2015) tarafından geliştirilen "Dijital Veri Güvenliği Farkındalığı Ölçeği"ni de içeren bir anket uygulanmıştır. Elde edilen verilere ilk olarak açıklayıcı faktör analizi yapılmış, ardından bazı değişkenlerin dijital veri güvenliği farkındalığı üzerindeki etkileri t-testi ve varyans analizi ile araştırılmıştır. Akıllı telefonunda antivirüs programı bulunanlarda dijital veri güvenliği farkındalığının daha yüksek olduğu tespit edilmiştir. Ayrıca, öğrencilerin ölçekteki ifadelere verdikleri yanıtların ortalamalarının üç civarında olduğu belirlenmiştir. Sonuç olarak, eczacılık öğrencilerinin bu konudaki farkındalıklarının geliştirilmesi gerekmektedir.

Anahtar Kelimeler: *Veri güvenliği, eczacılık uygulamaları, etik*

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INTRODUCTION

Usage of health information technology in healthcare is stated to be important, and it has benefits in costs, patient satisfaction, health outcomes (Rothstein, 2007; Sykes, Venkatesh, & Rai, 2011; Kuo, Ma, & Alexander, 2014). As time goes on, more data and information are available in the digital environment and can be easily stored, copied, and moved on small devices (Öğütçü, Testik, & Chouseinoglou, 2016). Today, health data are stored as electronic records by hospitals and clinics (Wang, 2015). There is a growing interest in using electronic health records instead of paper-based records (Fernández-Alemán, Señor, Lozoya, & Toval, 2013). It brings several advantages such as improving data availability, reducing costs and medical errors (Sun & Fang, 2010). The use of electronic health records improves patient care with immediate access and exchange of information, on the other hand, some risks as unauthorized access and use, raise concerns about confidentiality and privacy (Sulmasy, López, Horwitch, American College of Physicians Ethics, Professionalism and Human Rights Committee, 2017). The ethical principles of justice, autonomy, privacy, and confidentiality are considered in evaluating health technologies (Nesipoğlu & Özdinç, 2021). Electronic data security and information security management are sensitive issues for organizations (Parsons, McCormac, Pattinson, Butavicius, & Jerram, 2014). In the literature, the most cited concerns are privacy and security (Chao, Hu, Ung, & Cai, 2013; Sun & Fang, 2010; Lee, 2017; Haas, Wohlgemuth, Echizen, Sonehara, & Müller, 2011; Wang 2015; Yüksel, Küpçü, & Özkasap, 2017). Lack of security awareness threatens information security (Karabatak & Karabatak, 2019). As digital healthcare services increase around the world, the necessity of making regulations has arisen. European Union and the United States made regulations to protect health data and ensure privacy (Treacy & McCaffery, 2016). Similarly, in Turkey, Personal Data Protection Law, and Regulation on Personal Health Data entered into force in 2016 and 2019, respectively (Official Gazette, 2016, No:6698; Official Gazette, 2019, No: 30808).

Pharmacy is not apart from these developments and changes in healthcare. Various studies exhibit that health technologies have been taking part in pharmacy practice (Stewart & Lynch, 2012; Williams, Nunemacher, Holland, Rhodes, & Marciniak, 2019; Martirosov, Seitllari, Kaurala, & MacDonald, 2020; Westbrook et al., 2019; McNicol, Kuhn, & Sebastian, 2019; Burgin, O'Rourke, & Tully, 2014). Human errors are one of the major problems in ensuring information security, and raising awareness on this issue is essential (Çetinkaya, Güldüren, & Keser, 2017). In addition, considering the development, management, and usage processes, the importance of the human factor becomes evident (Karaoğlan Yılmaz, Yılmaz, & Sezer, 2014). In this regard, healthcare providers' responsibility and attention are important. Hence, increment of pharmacists' awareness about data security is essential.

Importance should be given to cybersecurity to protect health data (Wang, 2015). In a study conducted with physicians, lack of knowledge and skills are stated as significant challenges in using electronic health record systems (Chao et al., 2013). Privacy is at the forefront for people with high digital data security awareness levels (Durak & Saritepeci, 2020). In this context, improving healthcare professionals' skills, knowledge, and awareness is essential. In the literature, there are some studies about digital data security awareness. For instance, Avcı and Arslan (2019) conducted a study with employees of a public service organization and found a significant positive relationship between information literacy levels and digital data security awareness. Besides, Göldağ (2021) applied a study to university students and showed a significant positive relationship between digital literacy levels and digital data security awareness. Moreover, in Gündüzalp's (2021) study, the awareness

of university employees about digital data security and cyber security were determined.

Considering that pharmacists have ethical responsibilities to patients, and health information technologies are widely used, ensuring digital data security is essential for pharmacists. As future pharmacists, pharmacy students should pay attention to this issue. Within the scope of the study, the status of pharmacy students' digital data security awareness and the influences of some variables on digital data security awareness are examined.

MATERIAL AND METHODS

This study was approved by Izmir Katip Çelebi University Social Research Ethics Committee (Permit No: 2020/09-06). The questionnaire was applied online between November and December 2020 to pharmacy students in a pharmacy faculty. Informed consent was taken from the participants. In total 403 students exist in the faculty, and 88 students answered the questionnaire.

The questionnaire form consists of questions about gender, grade, internet usage, antivirus programs, and the Digital Data Security Awareness Scale developed by Yılmaz, Şahin, & Akbulut (2015). The scale has one dimension and five-point Likert type 32 items. The Cronbach's alpha coefficient of the scale is 0.945.

Data obtained from the study were analyzed with IBM SPSS 24.0 package program. Descriptive statistics were given. Besides, exploratory factor analysis was conducted on the data, Cronbach's alfa and Kaiser-Meyer-Olkin (KMO) values were calculated. T-test and variance analysis (ANOVA) were used to determine the effects of the variables on the factor. Also, the mean values of the items were investigated.

RESULTS AND DISCUSSION

The study results showed that most of the participants were female (62.5% female, 37.5% male). When examined according to the grades, 27.3% of the students were in their first year. 19.3%; %14.8%; 22.7% and 5.9% of the students were in the second, third, fourth, and fifth grade, respectively. In terms of internet usage in a day, 40.9% of the participants used for 3-5 hours, and 30.7% used for more than 5 hours. 23.9% used for 1-3 hours, 4.5% of them were used for less than 1 hour. The status of having an antivirus program on computers or smartphones was examined. The study results showed that half of the students (50%) have an antivirus program on their computers, and 34.1% do not have. 15.9% expressed that they don't have a computer. Most of the students (71.6%) don't have an antivirus program on their smartphones, while 28.4% have.

The KMO value was found as 0.913. Exploratory factor analysis was performed and limited to one factor, as indicated in Yılmaz, Şahin, & Akbulut's (2015) study. According to the results, the factor loadings of all expressions were found to be above 0.5, and the explained variance ratio was 56.4%.

The Cronbach's alpha coefficient of the scale was determined as 0.974. The factor loadings of the items were shown in Table 1. T-test and ANOVA analysis were performed to determine whether there is a difference according to gender, grade, internet usage, and the antivirus program on smartphones. Only those with an antivirus program on their smartphones had a statistically significant difference $p<0.05$, and their digital data security awareness was found to be higher.

Table 1. Factor Loadings

Items	Factor loadings
i18	0.86
i2	0.85
i15	0.85
i32	0.84
i14	0.83
i17	0.83
i11	0.82
i28	0.82
i22	0.81
i25	0.80
i19	0.80
i13	0.79
i26	0.78
i12	0.78
i30	0.77
i27	0.77
i8	0.76
i16	0.75
i7	0.74
i6	0.73
i24	0.72
i23	0.72
i21	0.71
i20	0.70
i9	0.70
i10	0.70
i3	0.68
i4	0.66
i29	0.65
i31	0.62
i1	0.54
i5	0.51

Table 2. Means ($\bar{x}$) and standard deviations (SD)

Items	Means ($\bar{x}$)	SD
i1	2.65	1.31
i2	3.83	1.34
i3	3.39	1.39
i4	3.66	1.43
i5	2.34	1.42
i6	3.09	1.45
i7	3.42	1.34
i8	3.56	1.48
i9	3.28	1.37
i10	3.06	1.40
i11	3.36	1.44
i12	3.36	1.34
i13	3.86	1.30
i14	3.86	1.32
i15	3.81	1.29
i16	3.57	1.41
i17	3.56	1.35
i18	3.83	1.35
i19	3.32	1.41
i20	3.31	1.43
i21	3.06	1.40
i22	3.24	1.34
i23	3.10	1.33
i24	4.15	1.36
i25	3.77	1.45
i26	3.58	1.36
i27	3.32	1.40
i28	3.76	1.27
i29	2.99	1.37
i30	3.82	1.34
i31	2.61	1.30
i32	3.28	1.34

In Table 2, the means and standard deviation values of the items were given. The items with the least and highest means were found to be 2.34 and 4.15. According to the results, items with the least and highest mean were about firewall software knowledge, and awareness in setting passwords for devices to prevent unauthorized access, respectively. The average mean of all items was 3.4.

The findings of this study revealed that gender, grade, or internet usage did not make a statistically significant difference in digital data security awareness of pharmacy students. However, having an antivirus program on smartphones makes a difference. The fact that the average of the items is around three indicates that the awareness of the pharmacy students should be increased.

In the literature, there are various studies investigating the role of gender in security awareness. In a study conducted with medical secretaries, information security awareness of females was found to be higher than males (Filik & Ünalan, 2021). Information security awareness of hotel managers did not differ according to gender (Okul, Şimşek, Hafçı & Barış, 2018). In a study applied to university students, no difference was found in cyber security behaviors by gender (Karacı, Akyüz, & Bilgici, 2017). Additionally, in employees' digital data security awareness, no significant gender difference was exhibited (Gündüzalp, 2021). Besides, studies exist indicating that digital data security awareness is higher in males (Göldağ, 2021; Yılmaz, Şahin, & Akbulut, 2016; Korkmaz, 2018). In this study, no statistically significant difference was found in digital data security awareness in terms of gender. Yılmaz et al. (2016) expressed that digital data security awareness of teachers increases when the daily internet usage time exceeds 3 hours a day. However, the present study results showed that no statistically significant difference was found in digital data security awareness according to daily internet usage. Parallel with this finding, there was no difference in the information security awareness of hotel managers in terms of internet usage times (Okul et al., 2018). Mylonas, Kastania, & Gritzalis (2013) found nearly one-quarter of smartphone users have security software on smartphones; however, most of them use security software on their computers, additionally presented that approximately one-third of the participants consider security software is not essential for smartphones. Similarly, in the present study, nearly one-third of pharmacy students use an antivirus program for their smartphones, and half of them have an antivirus program on their computers. Bıkmaz (2017) stated that approximately forty percent of health management department students have security software for smartphones, and nearly one-third use security software for laptops.

In literature, some studies applied the Digital Data Awareness Scale to university students. Göldağ (2021) stated that students have high digital data security awareness. Korkmaz (2018) found the statements' mean as generally close to four and the least mean as 3.30. However, in the present study, pharmacy students' answers to the statements were lower, and their awareness should be developed. Göldağ (2021) stated that students have the highest awareness about creating passwords for devices to prevent unauthorized use. Similarly, in the present study, pharmacy students have the highest awareness about it. Karaoğlan Yılmaz et al. (2014) applied a study with university students and presented that nearly half of them used username and password, and approximately one-tenth used firewall to prevent others from accessing their computer. The current study shows pharmacy students have a low level of knowledge about firewall software. In Okul et al.'s (2018) study, according to an open-ended question result, hotel managers were found not sufficiently knowledgeable about how a secure password should be. In the present study, the statements about passwords are over three.

CONCLUSION

Considering the security importance in healthcare, patient confidentiality, and privacy, future pharmacists' awareness of digital data security is vital. According to the study results, pharmacy students' awareness should be increased. It is thought that, in the light of current advancements, attaching more importance to this subject in the pharmacy curriculum will contribute to increasing the awareness of students. This study is applied to pharmacy students only. Thus for future studies, it may be conducted with pharmacists. Also, the qualitative methodology can be used for an in-depth examination. Seminars may be given to pharmacists and pharmacy students to increase awareness of this issue.

CONFLICT OF INTEREST

No conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Design, preparing the study text, reviewing the text, statistics, analysis and interpretation of the data, literature research. (NT)

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RESEARCH ARTICLE

Fabrication and Evaluation of Cationic Charged Magnetic Nanoparticles for Enhanced Gene Delivery

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Fabrication and Evaluation of Cationic Charged Magnetic Nanoparticles for Enhanced Gene Delivery

SUMMARY

Magnetofection; represents nucleic acid delivery by using magnetic nanoparticles (MNPs) under the influence of a magnetic field; gives promising results for gene delivery. However, pharmaceutical and biomedical studies in this area are very limited. To meet this need, we aimed to develop an effective magnetic gene delivery system in this study. The in-situ surface coating method was handled to develop cationic charged MNPs. Three different MNP formulations were obtained and investigated in terms of characterization, DNA binding, protection, and transfection ability. According to the results, the obtained MNPs have particles under 150 nm with a low PDI (<0.3), and positive zeta potential with a spherical shape. The DNA binding and protecting ability from nucleases were shown by agarose gel studies. No significant cytotoxicity was observed on COS-7 cells in the concentration range of 4-20 µL/well. Moreover, transfection studies revealed that the optimal system (GMS-MNP-1) showed significantly higher transfection efficacy comparing the naked plasmid or non-magnetic version of nanoparticle under a magnetic field (p>0.05). Promising results have been obtained with the use of obtained GMS-MNPs in terms of magnetic gene delivery. This work can be extended to in vivo by using disease-specific therapeutic genetic materials.

Key Words: *Gene delivery, magnetofection, cytotoxicity, transfection*

Gelişmiş Gen Teslimatı için Katyonik Yüklü Manyetik Nanopartiküllerin İmalatı ve Değerlendirilmesi

ÖZ

Manyetofeksiyon; manyetik alanın etkisi altında manyetik nanopartiküller (MNP'ler) kullanılarak nükleik asit aktarımı olarak tanımlanan, gen terapisi için umut verici sonuçlar veren bir tekniktir. Ancak bu alandaki farmasötik ve biyomedikal çalışmalar oldukça sınırlıdır. Bu ihtiyacı karşılanması için çalışmamıda etkili bir manyetik gen aktarım sistemi geliştirmeyi amaçladık. Katyonik yüklü MNP'lerin geliştirilmesi için yerinde yüzey kaplama yöntemi kullanıldı. Üç farklı MNP formülasyonu elde edildi ve karakterizasyon, DNA ile kompleks oluşturma, koruma ve transfeksiyon etkinliği açısından araştırıldı. Sonuçlara göre, elde edilen MNP'lerin 150 nm'nin altında, düşük PDI değerinde (<0.3) ve pozitif zeta potansiyelde küresel partiküller oldukları gösterildi. DNA ile kompleks oluşturma ve nükleazlardan DNA'yı koruma yeteneği, agaroz jel çalışmaları ile gösterildi. 4-20 µL/kuyucuk konsantrasyon aralığında COS-7 hücrelerinde önemli bir sitotoksisite gözlenmedi. Ayrıca, transfeksiyon çalışmaları, optimal olarak belirlenen sistemin (GMS-MNP-1), manyetik alan altında çıplak plazmit veya nanopartikülün manyetik olmayan versiyonuna kıyasla önemli ölçüde daha yüksek transfeksiyon etkinliği gösterdiğini ortaya koydu (p>0.05). Elde edilen GMS-MNP'lerin manyetik gen aktarımı açısından kullanılmasıyla umut verici sonuçlar elde edilmiştir. Bu çalışma, hastalığa özgü terapötik genetik materyaller kullanılarak in vivo olarak genişletilebilir.

Anahtar Kelimeler: *Gen aktarımı, manyetofeksiyon, sitotoksisite, transfeksiyon*

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INTRODUCTION

Nanotechnology covers the applications of new substances, materials, and methods developed by using nanometer-sized materials in medicine, engineering, pharmacy, physics, and many other sciences. It finds wider application areas, especially in molecular science and biomedicine (Kami et al., 2011). Since nanoparticles are small enough to easily enter almost all body areas, including the circulatory system and cells, they have become the basis of basic biomedical research, diagnostic science, and therapeutic applications of nanotechnology (Selmani et al., 2022).

In gene therapy, the therapeutic genes to be transferred have to deal with intracellular and extracellular barriers. Intracellular barriers originate from the cell, including the cell membrane, endosome, and nuclear membrane. On the other hand, extracellular barriers include serum proteins, nuclease enzymes, and the body's defense system. All these obstacles significantly reduce the efficiency of gene transfer (Sharma et al., 2021). Vector systems have been developed to overcome these barriers and ensure efficient gene transport (Foldvari et al., 2016).

Due to the safety and efficacy problems encountered in various gene transfer techniques, magnetic nanoparticle (MNP) applications have been oriented towards gene transfer over time. The beginnings of MNP-based transfection methods date back to the research of magnetically targetable drug delivery systems by Widdler et al in the late 1970s (McBain et al., 2008). This method is based on the principle that the therapeutic agent is attached to micro or nano-sized particle with magnetic properties or is entrapped in these particles. By modifying the surface properties of the MNP, it is possible to bind the drug or DNA on it and direct it to the target tissues and cells. Targeting a cytotoxic treatment agent directly to the chemotherapy site or directing the therapeutic DNA to the genetic disordered area are some examples of MNPs applications (Bi et al., 2020).

In many studies, it has been proven that gene transfer under a magnetic field by using MNPs; also called magnetofection; increases transfection efficiency (Kami et al., 2011). This technique is based on the binding of genetic material to MNPs. For MNP-based *in vitro* transfection, the particle-DNA complex is applied to the cell culture and a magnet or electromagnet is placed on the underside of this cell culture, which can generate a magnet-like electromagnetic field. Thus, the sedimentation and transfection rate of the DNA-particle complex increases (Dowaidar et al., 2017).

The total surface charge and the size of the MNP nucleic acid complex play essential roles in the cellular uptake of cells (Jin & Kim, 2014). For this purpose, cationic lipid coating strategies are applied to MNPs to form complexes with oppositely charged nucleic acids via electrostatic interaction, where nucleic acids are considered negatively charged molecules due to their phosphate groups (He et al., 2007; Song et al., 2010).

Many routes have been developed to synthesize MNPs, which are popular options in medical applications. In this study, the *in-situ* surface coating method was handled to develop cationic charged MNPs for gene delivery. Three different MNP formulation was obtained and investigated in terms of characterization, DNA binding, protection, and transfection ability.

MATERIAL AND METHODS

Green fluorescent protein-encoding plasmid (pEGFP-C1) was purchased from Invitrogen, USA. The pEGFP-C1 was amplified in the *DH5α* strain of *E. coli* and extracted by Gene Jet Endo-free plasmid maxiprep kit (Thermo Scientific, USA).

Glyceryl monostearate (GMS) and Kolliphor HS15 were obtained from BASF, Germany. Ethanol, Tween 80, $FeCl_{2,}$ and $FeCl_3$ were provided by Merck- Co. (Hohenbrunn, Germany). Cremephor RH40, Span 80, and dimethyl dioctadecyl ammonium bro-

mide (DDAB) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). XTT cell proliferation assay kit was obtained from Thermo Fisher Scientific (MA, USA). African green monkey kidney fibroblast-like cell line (COS-7) cell line was purchased from ATCC. All other chemicals were used as an analytical grade.

MNP Synthesis

Three different MNP formulations were developed by using the combination of microemulsion and multiple emulsion techniques. MNPs were synthesized in the core of multiple emulsions. GMS and DDAB were used as the main solid lipid and cationic lipid, respectively for all three formulations as the 1:1 weight ratio.

As the first step for magnetic nanoparticle preparation, the triangle phase diagram of water in oil (w_1/o) microemulsion was obtained at 10°C higher than the solid lipid melting point (Akbaba et al., 2017). Different surfactant and co-surfactant components used for magnetic nanoparticle synthesis was given in Table 1. Fe^{+2} and Fe^{+3} solutions were used as internal water phase (w_1/o) in all formulations. Transparent regions belonging to w_1/o microemulsion area were drawn in phase diagrams. DDAB was incorporated into the oil phase of the microemulsion for gaining cationic property to the MNPs. The obtained microemulsion was used as an interior emulsion of multiple emulsions (w_1/o/w_2). Then, by using an outer surfactant and ultrapure water, multiple emulsion was obtained. The pH of the system was increased by using 1 N NaOH and by leaking [OH^-] ions to the interior water phase of the multiple emulsion, magnetic iron oxide particles were synthesized in the core of cationic lipids (Schmidts et al., 2010).

Table 1. Compositions of developed magnetic nanoparticle formulations. (S_1:surfactant$_1$; Co-S:co-surfactant; S_2:surfactant$_2$; w/w: weight/weight)

Formulation code	GMS	DDAB	S_1	Co-S	S_1:Co-S (w/w)	S_2
GMS-MNP-1	+	+	S80:T80	EtOH	1:3	T80
GMS-MNP-2	+	+	S80:HS15	EtOH	1:3	HS15
GMS-MNP-3	+	+	S80:RH40	EtOH	1:3	RH40

As the last step for MNP development, 1 mL of hot multiple emulsion with magnetic core was dispersed into the ice-cold distilled water (0-2°C). MNPs were formed when multiple emulsion droplets met with cold water (Akbaba et al., 2018; Cavalli et al., 2000). A neodymium magnet was used for the separation of GMS-MNPs. GMS-MNPs were washed with UPH_2O two times and redispersed in UPH_2O. The final concentration was 25 mg/mL, with respect to solid lipids for all GMS-MNP formulations.

Characterization

Magnetic properties of GMS-MNPs were confirmed by using Lakeshore Vibrating Sample Magnetometer (VSM). Moreover, the particle size and zeta potential of nanoparticles were evaluated with the dynamic light scattering (DLS) method. For this purpose, Zetasizer Nano ZS (Malvern Instruments, Worcestershire, U.K.) was used. Experiments were carried out at least in triplicate. The morphology of GMS-MNPs was further evaluated by Transmission Electron Microscope (TEM, FEI Tecnai G2 Spirit BioTwin CTEM, Oregon, USA) for visualizing the samples.

Confirmation of complex formation ability

GMS-MNPs were complexed with pEGFP-C1 through electrostatic interactions between cationic lipids in the nanoparticles and anionic phosphate groups in the DNA. For this purpose, 3 µL GMS-MNPs (containing 25 mg/mL with respect to solid lipids) was added onto the 1 µL pEGFP-C1 solution (100 ng/µL) and incubated for 30 minutes on a benchtop shaker at 25 °C. Complex formation was checked by agarose gel electrophoreses. Agarose gel was prepared in 1 x tris-acetate-EDTA (TAE) buffer

as the final agarose concentration 1% (w/v) and the gel was run for 60 minutes at 100 V. To visualize; the gel was stained with ethidium bromide (0.5 µg/ml in UpH_2O) and photographed with UV transilluminator (Syngene, UV Transilluminator, USA).

Evaluation of protection ability

Another important parameter to achieving gene delivery is protecting the transported nucleic acids against nuclease enzymes. To evaluate this ability, GMS-MNPs and pEGFP-C1 were first complexed as explained above. Following the complex formation, 0.4 IU DNase I (New England Biolabs, USA) was added per each 1 µg DNA and incubated for 30 minutes in a 37 °C incubator to mimic body temperature (Capan et al., 1999; del Pozo-Rodríguez et al., 2009). At the end of the incubation period, SDS (1%) was added onto the complex to release the DNA (Erel-Akbaba & Akbaba, 2021). The released pEGFP-C1 was visualized by agarose gel electrophoreses and the degradation percentage was calculated via Image J software. As a control, GMS-MNPs were also complexed with the same amount of pEGFP-C1 and released by using SDS without incubating DNase I for all formulations.

Cytotoxicity profiles

Cytotoxicity profiles of obtained GMS-MNPs and GMS-MNP:pEGFP-C1 complexes were evaluated on COS-7 cell line. For this purpose, the cells were cultured in complete media containing Dulbecco's Modified Eagle's Medium (DMEM, low glucose) with 10% fetal bovine serum (FBS) and penicillin– streptomycin (100 UI/ml penicillin, 100 µg/ml streptomycin).

COS-7 cells were seeded into 96 well plates at the concentration $1x10^5$ cells/well and incubated overnight in a 5% CO_2 atmosphere at 37 °C. The following day, the medium was replaced with fresh DMEM that contains 4, 8, 12, 16, and 20 µL/well formulations and further incubated for 24 h. The highest three equivalent doses for each GMS-MNP were also evaluated for the complex forms in terms of cytotoxicity.

At the end of the incubation period, XTT cell proliferation assay kit protocol (Biological Industries, Israel) was performed by using Thermo Varioscan multiplate reader (Thermo, USA). The viability of cells was calculated by normalizing the fluorescence of untreated cells. Experiments were performed in quadruplicate.

Evaluation of transfection ability

The transfection ability of obtained formulations was evaluated on COS-7 cell line by using fluorescence microscopy and flow cytometry. For this purpose, cells were seeded in 6-well culture plates at a density of $5x10^4$ cells/ml and incubated overnight. To perform transfection, the 2.5 µg pEGFP-C1 plasmid was complexed with GMS-MNPs at the 3:1 volume ratio and applied to the cells. As a control group, the transfection ability of the naked pEGFP-C1 plasmid was also evaluated.

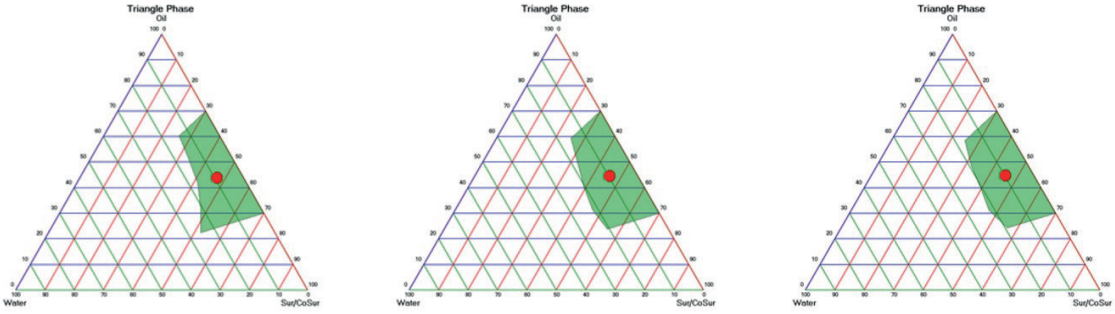
The cells were incubated for 4 h with formulations. At the end of the 4 h, the medium was removed and a fresh growth medium was added. The cells were allowed to grow and protein synthesis for a further 48 h. The transfection was first visualized by fluorescence microscopy (IX71, Olympus, Tokyo, Japan). Subsequently, flow cytometric analysis was also performed to detect the green fluorescence protein signal which occurs in the transfected cells. For flow cytometry, the cells were trypsinized and harvested, then washed twice with phosphate-buffered saline (PBS) and suspended in 100 µl FACs buffer (2% calf serum in PBS) Flow cytometry was performed by BD Accuri™ C5 (AZ, USA). Data corresponding to 10,000 events were collected for every group and analyzed with BD CFlow software (AZ, USA).

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). A non-paired t-test and one-way variance analysis (ANOVA) followed by multiple comparison tests were used to evaluate the statistical analysis between the groups. A p-value < 0.05 was considered as significant.

RESULTS AND DISCUSSION

The multiple emulsion technique was handled in this study to develop GMS-MNPs. For this purpose, (w_1/o) microemulsion phase diagrams were determined as given in Figure 1. According to the triangular diagrams, the formulation ratios contained the largest volume of inner water phase was selected and used for the formation of magnetic nanoparticles. To ensure the magnetic properties of the developed formulation, VSM studies were performed as well (Figure 2).



GMS-MNP-1 **GMS-MNP-2** **GMS-MNP-3**

Figure 1. Triangular phase diagram of w_1/o microemulsion formed with GMS as oil phase, Fe^{+2} and Fe^{+3} solutions as water phase, and various S/Co-S mixtures. The Green area shows a transparent w_1/o microemulsion region.

The regions of transparent microemulsion formation have a similar area for both formulations. The main parameter of this formation is that the HLB values are the same for each surfactant mixture which is equivalent to 5.58. Since the clear w_1/o microemulsion will then be used as the inner phase of the o/w_2 emulsion, it is important that it should be stable.

Following stable w_1/o microemulsion formation, the MNPs have formed as explained in the materials and methods section. The obtained MNPs were characterized in terms of size, polydispersity index (PDI), and zeta potential (Table 2). The ideal nanoparticle size may be different depending on the targeting tissue, for example, smaller size nanoparticle production is required to overcome the blood–brain barrier, as well as bigger nanoparticles, are needed for lung accumulation. According to the characterization results, the particle sizes of obtained GMS-MNPs were measured under 150 nm and PDI's were under 0.3 which is important for showing nanoparticles uniformity in terms of the size distribution (Elsana et al., 2019). Zeta potential values of GMS-MNPs were determined positive (above +20 mV) for all three formulations as expected. This charge is further used to complex pEGFP-C1 plasmid to the outer surface of nanoparticles through electrostatic interactions (Ozder & Akbaba, 2021). Moreover, TEM images supported that all GMS-MNPs are nano-sized and in globular structure (Figure 2).

Table 2. Characterization results for prepared MNP formulations as mean ± standard deviation (SD) (n=3).

Formulation	Particle size (nm ± SD)	PDI (± SD)	Zeta Potential (mV ± SD)
GMS-MNP-1	139.9 ± 2.9	0.160 ±0.023	41.1 ± 1.6
GMS-MNP-2	185.5 ± 1.5	0.158 ±0.011	20.7 ± 0.8
GMS-MNP-3	214.4 ± 2.7	0.255 ±0.076	20.2 ± 2.7
GMS-MNP-1:pEGFP-C1 (3:1, v/v)	427.7 ± 21.5	0.327 ±0.066	30.6 ± 1.07
GMS-MNP-2:pEGFP-C1 (3:1, v/v)	550,0 ± 36.8	0.659 ±0.062	37.8 ± 3.10
GMS-MNP-3:pEGFP-C1 (3:1, v/v)	397.7 ± 8.1	0.511 ±0.027	29.4 ± 3.05

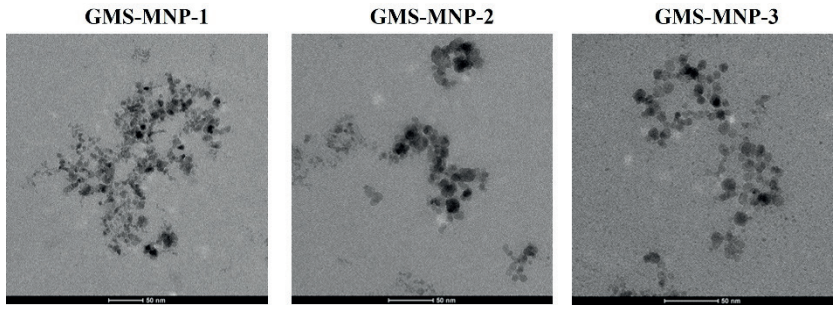


Figure 2. Morphological examination of GMS-MNPs. Scale bars represent 50 nm.

The magnetic properties of the nanoparticles obtained after the formation of the multiple emulsion and subsequent pH increase and washing were analyzed by VSM. An ideal magnetic nanoparticle should be paramagnetic (Gupta & Gupta, 2005; Tombácz et al., 2015). The magnetic properties such as saturation magnetization (Ms), remanent magnetization (Mr), and coercivity (Hc) were evaluated from the magnetization hysteresis of GMS-MNPs (Gupta & Gupta, 2005; Tombácz et al., 2015; Zhi et al., 2006).

As seen in Figure 3, all three formulations are in paramagnetic behavior and Mr and Hc values are approximately zero. According to the literature, saturation magnetization of magnetite is higher than in the coated samples we prepared. However, GMS-MNPs have reasonably sufficient Ms values for magnetic targeting of nucleic acids (Sun et al., 2008). Ms values were measured as 15, 10, and 8 emu g^{-1} for lipid-coated GMS-MNP-1, GMS-MNP-2, and GMS-MNP-3, respectively.

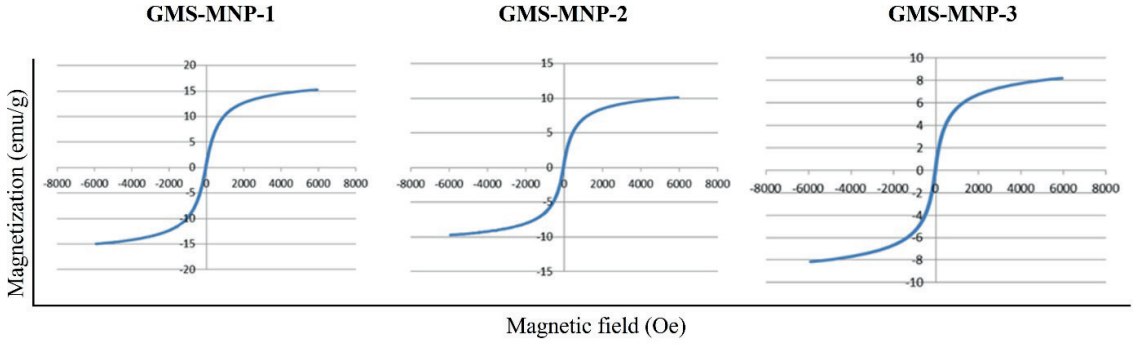


Figure 3. Magnetization hysteresis loops of GMS-MNP-1, GMS-MNP-2 and, GMS-MNP-3.

Agarose gel electrophoresis studies were carried on to confirm the complex formation via pDNA and determine the protection ability of the formulation against the degradation of nucleases (Figure 4). All three GMS-MNPs were interacted electrostatically with the pEGFP-C1 plasmid and blocked the DNA mobility on agarose gel electrophoresis at a ratio of 3:1 (v/v). The obtained GMS-MNP-1:pEGFP-C1 (3:1, v/v) were further characterized. As seen in Table 2, the particle sizes and PDI values of the complexed formulations were increased. This was an expected result, as DNA binds to the outer surface of the nanoparticles. On the other hand, the zeta potential remained positive. This may be adventageus for transfection as the positively charged nanoparticles could easily interact with the negatively charged cell membrane (Gan et al., 2005).

Stereological protection of the particles against the degradation of the DNase enzyme is required for

an effective transfection (Capan et al., 1999; del Pozo-Rodríguez et al., 2010). First of all, the efficacy of SDS in releasing pDNA from the complex was evaluated. As seen in Figure 4b, the pEGFP-C1 was successfully released from the complex with the help of SDS, for all formulations.

Figure 4c shows the results of the gel electrophoresis study involving the samples treated with DNase I. The bands were observed and the integrity of the GMS-MNPs were compared with a control of untreated pEGFP-C1 in lane 1 and naked pEGFP-C1 was threatened with the same amount of *DNase I* as control of enzyme activity in lane 2. The band densities were quantified with ImageJ Software and degradation percentages were calculated according to the control groups. Naked pEGFP-C1 was totally degraded after *DNase I* treatment. However, GMS-MNPs were protected pEGFP-C1 in various levels. The non-degraded pEGFP-C1 percentage after complex formation with GMS-MNP-1 was calculated as 71.7 %, for GMS-MNP-2 it was calculated as 77.9 %, and for GMS-MNP-3 it was calculated as 23.5 %.

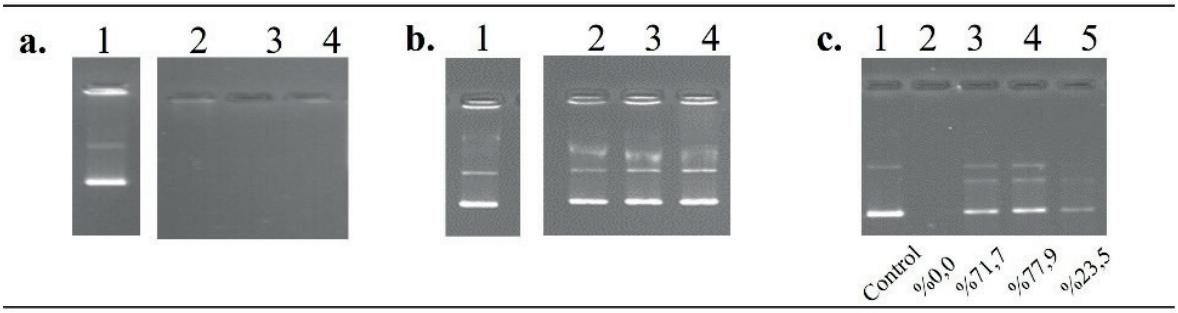


Figure 4. Gel retardation and DNase I protection studies agarose gel images. **a.** Confirmation of complex formation ability of GMS-MNP-1,2, and 3 (Lanes from left 1: naked pEGFP-C1 (100 ng/well); 2-4: GMS-MNP-1, 2, and 3 :pEGFP-C1 (3:1, v/v) complexes). **b.** Efficacy of SDS to release pEGFP-C1 from GMS-MNP: pEGFP-C1 complexes (Lanes from left 1: naked pEGFP-C1 (100 ng/well); 2-4: released pEGFP-C1 from GMS-MNP-1, 2, and 3 :pEGFP-C1 (3:1, v/v) complexes). **c.** DNase I degradation of GMS-MNPs. (Lanes from left 1: naked pEGFP-C1 (100 ng/well); 2: naked pEGFP-C1 + *DNase I* enzyme; 3–5: GMS-MNP-1, 2, and 3 complexes + *DNase I* enzyme).

Non-cytotoxic behavior of the developed system is another critical factor for formulation development studies. (Abas et al., 2021; Erel-Akbaba et al., 2020). According to the results of the cytotoxicity assay, no significant cytotoxicity was observed on COS-7 cells in the concentration range of 4-20 µL/well for GMS-MNP-1 and GMS-MNP-3 formulations (Figure 5). A dose-dependent decrease in cell viability was observed for GMS-MNP-2 formulation. The highest three equivalent doses for each GMS-MNP were also evaluated for each complex form in terms of cytotoxicity and the viability of the cells dramatically increased for the GMS-MNP-2 formulation. Considering the stereological protection and cytotoxicity studies, the GMS-MNP-1 formulation was determined to be superior to the GMS-MNP-2 and GMS-MNP-3 formulations in both conditions. However, the efficiency of transfection is one of the most important parameters to evaluate.

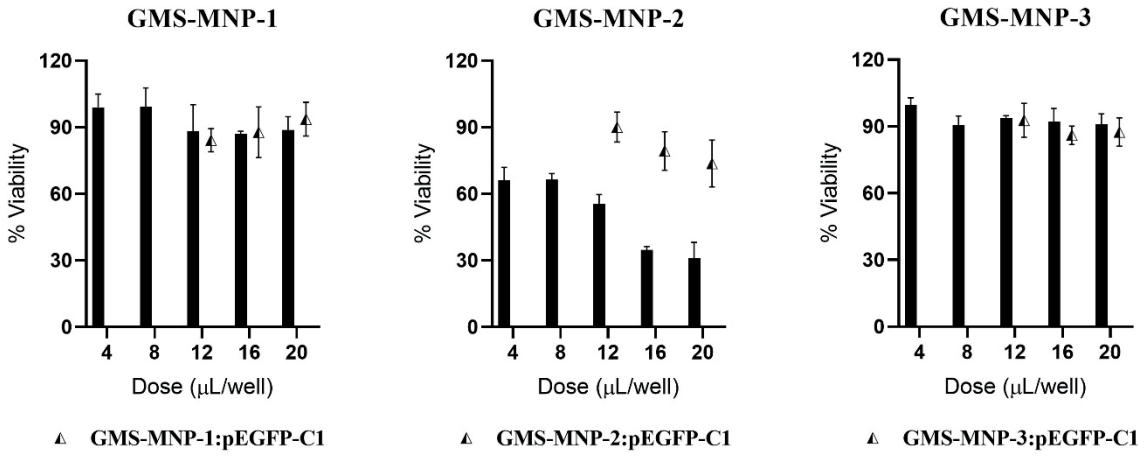


Figure 5. Cytotoxicity evaluation of GMS-MNPs and GMS-MNP:pEGFP-C1 (3:1, v/v) complexes

The ability and efficiency of transfection were evaluated both qualitatively and quantitatively. EGFP, which is the expression product of the pEGFP-C1 plasmid, has a single excitation peak centered at about 488 nm, with an emission peak wavelength of 509 nm excitation peak (del Pozo-Rodríguez et al., 2010). EGFP expressed by cells was visualized under an appropriate filter using fluorescence microscopy (IX71, Olympus, Tokyo, Japan) (Figure 6). Commercially available magnetofection agent PolyMag was used as a positive control. In order to observe the effect of magnetic field on transfection, the formulation containing UPH_2O instead of Fe solution in the inner aqueous phase was also added to the experimental protocol as a control together with naked pEGFP-C1 plasmid. According to fluorescence microscope images, although it is relatively higher in the GMS-MNP-1 formulation, similar levels of EGFP expression were observed in the GMS-MNP-2 and GMS-MNP-3 formulations, as well as PolyMag. The non-magnetic version of GMS-MNP-1 (called as GMS-NPS-1) formulation showed a significantly low level of transfection and no fluorescence signal was observed with the naked pEGFP-C1 ($p<0.05$). It can be deduced from the fluorescence microscopy results that the application of a magnetic field increases the transfection ability.

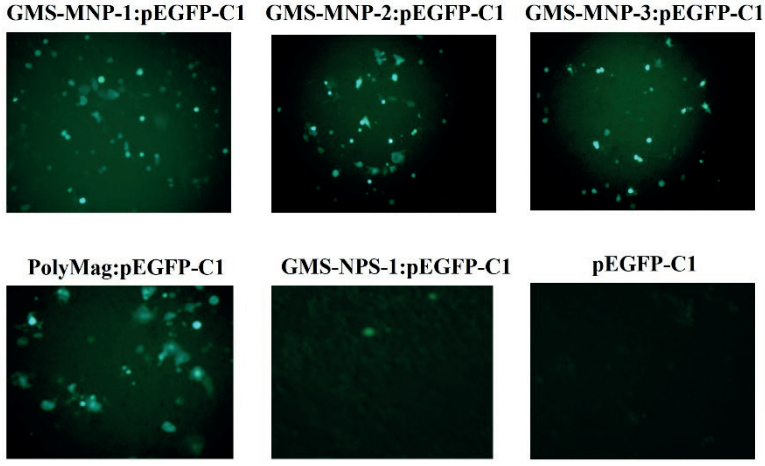


Figure 6. Qualitative magnetofection ability of GMS-MNPs, GMS-NP, and naked pEGFP-C1 plasmid. Images of EGFP-positive cells under a fluorescence microscope.

In order to demonstrate the transfection efficiency quantitatively, flow cytometry was performed under the blue laser (Figure 7). Obtained results are in parallel with the fluorescence microscopy images. Transfection efficiency was measured as 30.1% for GMS-MNP-1, 23.0% for GMS-MNP-2, and 26% for GMS-MNP-3. The transfection efficiency of GMS-MNP-1 is similar to the commercial product PolyMag (30.2%) used as a positive control. The transfection efficiency was found to be significantly lower for naked pEGFP-C1 (4.9 %).

Briefly, MNP-GMS-1 formulation is suggested as an optimal formulation considering its characteristics, stereological protection, toxicity, and magnetofection efficiency comparing MNP-GMS-2 and 3.

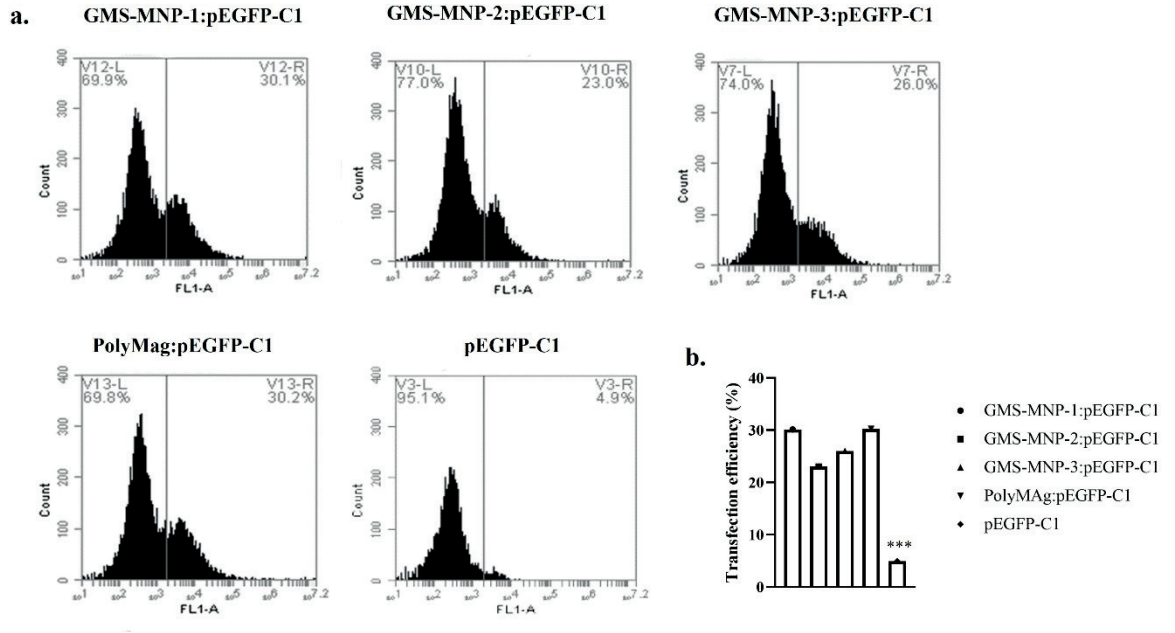


Figure 7. Representative flow cytometry histograms of transfection study groups (a). Quantitative analysis of EGFP expression percentage in COS-7 cells by flow cytometry analysis (b).

Targeted therapy with magnetic nanoparticles is an intriguing subject. As the novel genetic mechanisms were identified, the need for targeting these structures in the tissues or cells is increasing proportionally (Aslam et al., 2022). Magnetofection, one of the active drug targeting methods, attracts the attention of researchers as an important method to overcome this challenge.

In recent years, various studies on MNP-mediated gene targeting, especially cancer cell targeting, have been published. In one of these, polyethyleneimine-coated magnetic nanoparticles were designed to silence the overexpressed MUC1 gene in breast cancer. The developed MNPs have a particle size of 200 nm to 400 nm, similar to developed GMS-MNPs in our study (Amani et al., 2021). Another study focused on magnetic nanoparticles to target CRISPR/dCas9 ribonucleoproteins. MNP were coated with chitosan to deliver the CRISPR/dCas9 ribonucleoproteins, however, particle size of the developed system reached micron sizes (Lee et al., 2021). In another study, researchers investigated the performance of microcarriers as drug delivery systems that were applied to each branch of the lung under the influence of a magnetic field in terms of delivery of the drugs after the respiratory syndromes of Covid-19 (Ebrahimi et al., 2021). Although MNP synthesis using emulsion technology is a known method, it should be noted as a

novelty that no study has been encountered that provides lipid coating with in situ MNP synthesis.

CONCLUSION

To sum up, GMS-MNP formulations with 3 different surfactant compositions were developed and evaluated in parallel. Obtained GMS-MNPs are paramagnetic and have the ability to target genetic material under a magnetic field. However, GMS-MNP-1 formulation is suggested as an optimal formulation considering its characteristics, stereological protection, toxicity, and magnetofection efficiency in comparison to GMS-MNP-2 and GMS-MNP-3. Expanding studies on therapeutic genetic material delivery under the magnetic field and evaluating its *in vivo* potential are the future goals of this study.

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CONFLICT OF INTEREST

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

No Competing interests are at stake and there is No Conflict of Interest" with other people or organizations that could inappropriately influence or bias the content of the paper.

AUTHOR CONTRIBUTION STATEMENT

All authors have given approval for the final version of the manuscript. H.A. and G.E-A. carried out the experiments. H.A. and G.E-A. wrote the manuscript with support from A.G.K. H.A. conceived the original idea. A.G.K. supervised the project. All authors contributed to this work presented in this manuscript.

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RESEARCH ARTICLE

A Cosmetic Nanoemulsion Against Seborrheic Dermatitis: Development, Characterization and Effectiveness

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A Cosmetic Nanoemulsion Against Seborrheic Dermatitis: Development, Characterization and Effectiveness

SUMMARY

In this study, it was aimed to develop a topically applicable nanoemulsion (NE) that is expected to have an ameliorating effect in seborrheic dermatitis (SD). The main purpose of the formulation is to eliminate the disease factor, to repair the damage caused by the disease on the skin and to smooth the skin appearance by moisturization. For this reason, in vitro antimicrobial effect and in vivo effectiveness of the formulation were tested. For this aim; essential oils from tea tree, sage, cinnamon, oregano; extracts from Aloe vera, colloidal oatmeal, liquorice; vegetable oils from grape seed and sesame, and honey were used in a NE formulation. The NEs were prepared by ultrasonication method. Preliminary stability tests were applied to all formulations and then, pH, conductivity, viscosity, average droplet size, polydispersity index (PDI), and zeta potential measurements were taken on the selected NEs for 3 months. Finally, the antimicrobial effect and in vivo effectiveness of the optimum NE were tested. The average droplet size, PDI, and zeta potential value of the optimum formulation (F6P2) were 108.40 ± 0.90 nm, 0.195 ± 0.07, and -21.40 ± 1.45 mV, respectively. As a result, the moisture content of the skin increased significantly ($p < 0.001$), the sebum and redness values significantly decreased ($p = 0.008$ and 0.001, respectively) and there was no significant change in the pH of the volunteers' skin. Accordingly, it can be concluded that the optimum NE formulation developed in this study may be beneficial as a supplement for patients with SD.

Key Words: *Seborrheic dermatitis, nanoemulsion, herbal cosmetic, efficacy tests, essential oils, plant extracts, vegetable oils, honey*

Seboreik Dermatite Karşı Kozmetik Bir Nanoemülsiyon: Geliştirme, Karakterizasyon ve Etkinlik

ÖZ

Bu çalışmada seboreik dermatitte (SD) iyileştirici etkisi olması beklenen topikal olarak uygulanabilir bir nanoemülsiyon (NE) geliştirilmesi amaçlanmıştır. Formülasyonun temel amacı hastalık faktörünü ortadan kaldırmak, hastalığın ciltte verdiği hasarı onarmak ve nemlendirerek cilt görünümünü pürüzsüz hale getirmektir. Bu nedenle formülasyonun in vitro antimikrobiyal etkisi ve in vivo etkinliği test edilmiştir. Bu amaç için NE formülasyonunda; çay ağacı, adaçayı, tarçın, kekik esansiyel yağları; Aloe vera, kolloidal yulaf ezmesi, meyan kökü ekstreleri; üzüm çekirdeği ve susamdan elde edilen bitkisel yağlar ve bal kullanılmıştır. NE'ler ultrasonikasyon yöntemiyle hazırlanmıştır. Tüm formülasyonlara ön stabilite testleri uygulanmış ve ardından seçilen NE'ler üzerinde 3 ay boyunca pH, iletkenlik, viskozite, ortalama damlacık boyutu, polidispersite indeksi (PDI) ve zeta potansiyel ölçümleri alınmıştır. Son olarak, optimum NE'nin antimikrobiyal etkisi ve in vivo etkinliği test edilmiştir. Optimum formülasyonun (F6P2) ortalama damlacık boyutu, PDI ve zeta potansiyel değeri sırasıyla 108,40 ± 0,90 nm, 0,195 ± 0,07 ve -21,40 ± 1,45 mV olarak bulunmuştur. Sonuç olarak, cildin nem içeriği önemli ölçüde artmış ($p < 0,001$), sebum ve kızarıklık değerleri önemli ölçüde azalmış (sırasıyla $p = 0,008$ ve 0,001) ve gönüllülerin cildinin pH'ında önemli bir değişiklik meydana gelmemiştir. Buna göre, bu çalışmada geliştirilen optimum NE formülasyonunun SD'li hastalar için ek olarak faydalı olabileceği sonucuna varılabilir.

Anahtar Kelimeler: *Seboreik dermatit, nanoemülsiyon, bitkisel kozmetik, etkinlik testleri, uçucu yağlar, bitki ekstreleri, bitkisel yağlar, bal*

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INTRODUCTION

Seborrheic dermatitis (SD) is a common chronic recurring inflammatory disease that most commonly affects adults; however, a more transient infantile form also occurs (Del Rosso, 2011). SD is characterized by the presence of scratchy, erythematous areas with easily detachable, oily large scales. Although it can occur in a variety of anatomical locations, it tends to occur in areas containing multiple sebaceous glands, such as the scalp, face (nasolabial folds, ears, and eyebrows), upper chest, and back. Dandruff, a milder rash usually characterized by smaller dry, flaky scales, may occur on the scalp with SD (Berk & Scheinfeld, 2010). In some patients with SD, inflammatory erythematous folliculitis (possibly due to *Malassezia*) and blepharitis may develop. *Malassezia* species are thought to be the main factor in the development of SD. *Malassezia* species have been identified, seven of which have been associated with the human skin flora and SD. *Malassezia furfur, Malassezia pachydermatis, Malassezia restricta, Malassezia sympodialis, Malassezia globosa, Malassezia obobtusa* and *Malassezia slooffiae* have been detected in the affected skin (Vijaya Chandra, Srinivas, Dawson Jr, & Common, 2021).

Several treatment options can be effective in treating SD. The mechanism of action of the most common treatments includes inhibition of yeast colonization on the skin, reduction of itching and erythema, relaxation of crusts and scales, and reduction of inflammation (Berk & Scheinfeld, 2010). Mainstays of treatment for SD are antifungals, corticosteroids, calcineurin inhibitors, among other agents (Berk & Scheinfeld, 2010; Clark, Pope, & Jaboori, 2015; Gary, 2013). Current topical antifungals are largely safe and effective, but at times may be ineffective due to the increasing resistance of *Malassezia* to antifungal agents and some of these agents can cause irritant and allergic skin reactions. Moreover, even when their use is successful, there is still a high rate of recurrence in superficial skin diseases (Pintas & LIO, 2018). SD can also be treated with low or medium potency topical corticosteroids or calcineurin inhibitors (e.g., tacrolimus and pimecrolimus). These immunomodulatory agents are highly effective as anti-inflammatory agents, but long-term and prophylactic use seem to be inappropriate because of the chronic and recurrent nature of SD. Although steroids show efficacy and safety in short-term treatments, they could lead to frequent relapses shortly after the treatment discontinues. Also, they have the risk of causing addiction and steroid rosacea (du Vivier, 1976; Vardy, Cohen, Tchetov, Medvedovsky, & Biton, 1999; Rigopoulos, Ioannides, Kalogeromitros, Gregoriou, & Katsambas, 2004). Therefore, it may be important to identify novel treatments that may be more appropriate for long-term use without risk of these side effects (Pintas & Lio, 2018).

Natural ingredients with anti-*Malassezia* activity, have been anticipated to ensure patients with a wide range of treatments. Compliance is one of the main factors in the treatment of chronic conditions such as SD. Therefore, formulations containing natural ingredients with anti-*Malassezia* activity can be a prominent part of long-term treatment as an alternative and also for complementary management. In addition, it would be satisfactory to use them prophylactically in SD patients with mild symptoms (Han et al., 2017).

There are numerous advantages of using formulations containing natural ingredients for the treatment of SD, including patient compliance, fewer side effects, easy availability, low cost, and multiple modes of pharmacological action (Herman & Herman, 2016). Therefore, in this study, we developed a topical non-pharmacological formulation in NE form to be an alternative for synthetic drugs in SD therapy and to improve clinical outcomes of the disease. Essential oils from tea tree, sage, cinnamon, oregano; extracts from *Aloe vera*, colloidal oatmeal, liquorice; vegeta-

ble oils from grape seed and sesame, and honey were used in the NE formulation.

Tea tree oil is essential oil from the *Melaleuca alternifolia* tree, which is native to Australia (Beheshti Roy et al., 2014). This oil has been traditionally used for treatment of burns, infections and SD (Chandler & Osborne, 1998; Aburjai & Natsheh, 2003). Satchell et al. studied 5% tea tree (TT) oil to 63 patients with SD and observed a 41 percent improvement in symptom severity score compared to only 11 percent in the placebo group (Satchell, Saurajen, Bell, & Barnetson, 2002).

Essential oil of *Salvia officinalis* L. (sage) was shown to have antifungal activity against dermatophyte strains. Nitric oxide production stimulated by lipopolysaccharide in macrophages was inhibited by the essential oil without affecting cell viability, in concentrations up to 0.64 μL/mL. Consequently, it has been proposed as an appropriate bioactive agent in antifungal therapy (Abu-Darwish et al., 2013).

It has been confirmed that *Cinnamomum zeylanicum* (cinnamon) essential oil can be effectively down-regulated IL-1α , IL-6, IL-8 cytokines overproduced by the SD-inducing cocktail. Additionally, it also showed inhibitory effect on sebum lipid synthesis from primary sebocyte and growth inhibitory effect to Malassezia globosa yeast. As a result, *Cinnamomum zeylanicum* essential oil suggested as a natural herbal remedy to relieve or protect scalp SD (Kim, Kim, Lee, Jeon, & Park, 2012).

The essential oil from *Origanum vulgare* L. (oregano) possess strong inhibitory effects against a variety of phytopathogenic fungi and do not seems to cause the development of resistance in microorganisms (Vinciguerra, Rojas, Tedesco, Giusiano, & Angiolella, 2019). In a study conducted by Vinciguerra et. al., the antifungal activity of *Origanum vulgare* and *Thymus vulgaris* essential oils and carvacrol against 27 clinical isolates of *Malassezia furfur* were reported. Essential oils and carvacrol were found to be more actives against resistant or dose dependent to fluconazole *Malassezia furfur* isolates (Vinciguerra et al., 2019).

The compounds found in *Aloe vera* gel contain polysaccharides that are capable of reducing and restoring inflammation. It also has antimicrobial properties (Fozouni, Taghizadeh, & Kiaei, 2018). In a study with 44 patients with SD, 24 adults used an emulsion containing 30% *Aloe vera* extract, whereas 20 volunteers applied a placebo emulsion. After a four-week application period, there was a significant decrease in flaking (36.6% treatment vs. 17.6% placebo) and itching (21.5% vs. 5.3%) when compared with baseline. In addition, 58% of the patients had complete resolution or significant improvement in SD symptoms (Vardy et al., 1999).

Avena sativa (colloidal oatmeal) has been used for centuries to decrease itching in a variety of xerotic dermatoses (Sur, Nigam, Grote, Liebel, & Southall, 2008). Studies have revealed that the dermal formulations of natural colloidal oatmeal, especially avenanthramide, improves disease symptoms by restoring the cutaneous barrier. It may also play an important role in reducing the use of corticosteroids and calcineurin inhibitors in atopic dermatitis (Pigatto et al., 1997; Eichenfield, Fowler Jr, Rigel, & Taylor, 2007; Cerio et al., 2010).

Glycyrrhiza glabra (liquorice*)* has significant anti-inflammatory and anti-allergic activity. Glycyrrhizin, a kind of flavone found in licorice, reinforces cortisol's inhibition of antibody formation, stress reaction, and inflammation (Saeedi, Morteza-Semnani, & Ghoreishi, 2003).

Grape seed oil comes from the seeds of *Vitis vinifera.* It is rich in phenolic compounds, fatty acids, and vitamins. Grape seed oil has beneficial properties for health that are mainly detected by *in vitro* studies, such as anti-inflammatory, cardioprotective, antimicrobial, and anticancer properties. Phytosterols pres-

ent in grape seed oil which may prevent the release of proinflammatory mediators by oxidized low-density lipoprotein-stimulated macrophage during oxidative stress and eicosanoid synthesis (Garavaglia, Markoski, Oliveira, & Marcadenti, 2016; Lin, Zhong, & Santiago, 2018).

Sesame oil contains powerful, natural antioxidants (sesamin, sesamol, sesamolin, and phytosterol) which give the oil very good oxidative stability. It has pronounced regenerative action due to its high unsaponifiable content. Sesame oil has been reported to have healing effects on certain skin conditions such as seborrhea, eczema, psoriasis and sunburn (Rabasco Álvarez & González Rodríguez, 2000).

Honey has antibacterial, antifungal and antioxidants activities and has high nutrient value. In a clinical trial, crude honey has been demonstrated to improve SD and associated hair loss and prevent relapse when applied weekly (Al-Waili, 2001).

To develop innovative and superior dermatological and/or cosmetic skin care products containing bioactive compounds, it is necessary to depart from the basic technology and use key technologies. Recent advances in nanotechnology are promising for the potential use of poorly soluble, poorly absorbed and unstable plant extracts and phytochemicals in cosmetics. A modern perspective can improve both the aesthetics and effectiveness of a cosmetic product. The application of new techniques can also increase the effectiveness of herbs regarding their sustained effect on the human body (Altuntaş, Yener, & Özkan, 2019).

One of the key technologies that will provide innovative products is NE. Nanoemulsions (NEs) can also be termed as ultrafine emulsions, miniemulsions, and submicron emulsions due to the formation of droplets in the submicron range. The average droplet size of NEs can range from 20 to 500 nm (Guglielmini, 2008). The small size of the droplets gives them a natural stability against creaming, sedimentation, and aggregation, while ensuring effective delivery of active ingredients to the skin (De Vleeschauwer & Van der Meeren, 1999; Fang, Hong, Chiu, & Wang, 2001). NEs have attracted considerable attention in recent years for application in personal care products as potential vehicles for the controlled delivery of cosmetics and the optimized dispersion of active ingredients in particular skin layers (Guglielmini, 2008). The simplicity of NE fabrication also attracted interest towards increased loading, improved therapeutic efficacy and stability of herbal drugs as compared to conventional delivery systems (Harwansh, Deshmukh, & Rahman, 2019). Ultrasonic emulsification is a high energy method to develop NEs. It utilizes sound waves with frequency more than 20 kHz by using a sonotrode to cause mechanical vibrations followed by the formation of acoustic cavitation. Collapse of these cavities generates powerful shocks waves which breaks the coarse droplets (Ghosh, Mukherjee, & Chandrasekaran, 2013). Recent studies have shown that, in emulsification process, ultrasound had emerged as an excellent yet superior tool as compared to rotor stator in terms of obtaining a smaller droplet size and high energy efficiency (Abismaıl, Canselier, Wilhelm, Delmas, & Gourdon, 1999; Kentish et al., 2008). In addition, the results showed that the amount of surfactant required to produce an emulsion of the desired diameter was significantly reduced and the energy consumption was considerably lower than with other conventional mechanical devices (Tadros, Izquierdo, Esquena, & Solans, 2004). More interestingly, all of the acoustically formed emulsions are perfectly stable and more homogeneous compared to mechanical methods (Abismaıl et al., 1999; Tadros et al., 2004).

In light of the above, we aimed to develop a dermal NE formulation that contains the combination of oils and extracts of medicinal plants and honey for providing synergistic or potentiating effects in

the treatment of SD. For this purpose, dermatological NEs containing essential oils from tea tree, sage, cinnamon, oregano; extracts from *Aloe vera*, colloidal oatmeal, liquorice; vegetable oils from grape seed and sesame, and honey were prepared using ultrasonic emulsification method. The prepared NEs were characterised and physical stability of them was evaluated for 3 months. Its antimicrobial activity was tested on *Candida albicans* (*C.albicans*) strains. Finally, the optimized NE was tested clinically in terms of the efficacy and safety in the treatment of SD with non-invasive biophysical techniques and subjective assessment.

MATERIAL AND METHODS

Materials

Cinnamon and oregano essential oils were purchased from Arifoğlu Inc. (Turkey). Sage essential oil was provided from Vitarom Chemical Co. Ltd. (Turkey). Extracts of *Aloe vera*, colloidal oatmeal and liquorice and tea tree essential oil were supplied by Surya Chemical Co. Ltd. (Turkey). Flower honey were obtained from Balparmak (Turkey). Grape seed oil and sesame oil were kindly donated from Zade Naturel (Turkey). Poloxamer 188 and tocopherol acetate were obtained from Sigma (St. Louis, MO). Transcutol® HP, Labrafac™ CC were purchased from Gattefossé (France). Cremophor® A25 was provided from BASF (Germany). Neolone™ PE was acquired as a gift from Kale Kimya Inc. (Turkey). All other chemicals and solvents used were of pure analytical grade.

Preparation of Nanoemulsions

NEs containing oily phase with 3 different concentrations (%5, %6.68, %10 (w/w)) were prepared by ultrasonic emulsification method. Two different surfactant and cosurfactant mixtures (Poloxamer 188/ Transcutol® HP and Cremophor® A25/Transcutol® HP) with 1:1 (w/w) and 1:2 (w/w) surfactant and cosurfactant ratios were evaluated to find a stable NE formulation. Poloxamer 188 (5% w/w) and Transcutol® HP (10% w/w) was the only surfactant and cosurfactant combination that resulted in a stable formulation and therefore was chosen for further study. The formulations and manufacturing parameters of the selected NEs (1:2 (w/w) surfactant to cosurfactant ratio) are given in Table 1.

Initially, the ingredients of water phase and oily phase were weighed precisely in two separate beakers. Coarse emulsion was manufactured with/without pre-emulsification stage before ultrasonication process. During pre-emulsification, coarse emulsion was obtained using a rotor-stator type mixer (Witeg HG-15D, Germany) for 5 minutes at a mixing speed of 8100 rpm. The emulsion was then ultrasonically emulsified using a 20 kHz Sonicator (Ultrasonics, USA) (750W Ultrasonic power supply). The energy input was delivered via a sonotrode containing a piezoelectric crystal with a probe diameter of 13 mm. The ultrasonication process was started after the sonicator probe was placed vertically in the coarse emulsion. The process was applied with 50% amplitude, 30 s pulse on and 30 s pulse off, for two different sonication periods, 20 minutes or 30 minutes. To control the temperature of the emulsion during sonication, the beaker was placed in a larger container containing ice. Subsequently, characterization studies of formulated NEs were carried out and also the stability of selected NEs was investigated.

Table 1. The formulation compounds and the manufacturing parameters of the selected NEs.

	Ingredients	FORMULATION CODES		
	(% w/w)	F4 (P1-P4)	F5 (P1-P4)	F6 (P1-P4)
OIL PHASE	Tea tree essential oil	1	0.56	0.33
	Sage essential oil	1	0.56	0.33
	Oregano essential oil	1	0.56	0.33
	Cinnamon essential oil	0.50	0.28	0.17
	Grape seed oil	2	1.1	0.67
	Sesame seed oil	2	1.1	0.67
	Neolone™ PE	0.30	0.30	0.30
	DL-alpha tocopherol acetate	0.20	0.20	0.20
	Transcutol® HP	10	10	10
	Labrafac™ CC	2	2	2
AQUEOUS PHASE	Poloxamer 188	5	5	5
	Colloidal oatmeal extract	2	2	2
	Aloe vera extract	0.5	0.5	0.5
	Liquorice extract	2	2	2
	Honey	4	4	4
	Distilled Water (d.w.)	66.50	69.82	71.50
PROCESS	**Procedure 1 (P1):** 20 min sonication without pre-emulsification **Procedure 2 (P2):** 20 min sonication with pre-emulsification (5 min at 8100 rpm) **Procedure 3 (P3):** 30 min sonication without pre-emulsification **Procedure 4 (P4):** 30 min sonication with pre-emulsification (5 min at 8100 rpm)			

Characterization of Nanoemulsions

Particle Size Analysis

The mean droplet size and PDI of the produced NEs were measured by quasi-elastic light scattering technique based on the measurement of Brownian motion (movement in random direction) of sub-micron particles as a function of time. Light scattering was monitored at room temperature (25°C) at a scattering angle of 90° by Malvern Zetasizer Nano ZS (Malvern Instruments, UK). Samples of NEs were properly diluted with distilled water (1:100 v/v) prior to analysis to reduce the multiscattering effect (Rebolleda et al., 2015). Samples were considered polydisperse when the PDI was higher than 0.3 (He et al., 2011).

Zeta Potential Measurement

Zeta potential is measured using the aforementioned Malvern Zetasizer Nano ZS device. To measure zeta potential value of the NEs, samples were diluted with distilled water (1:100 v/v) and its value is estimated from the electrophoretic mobility of oil droplets (Rebolleda et al., 2015).

Rheological Characteristics

The viscosity of the NEs at different shear rates was studied using Brookfield cone and plate viscometer (Brookfield Engineering Laboratories, Inc., Mid-

dleboro, MA) at 25 ± 0.5 °C. The graphs were plotted between viscosity versus shear rate to evaluate rheological characteristics of the NEs (Shakeel, Ramadan, & Ahmed, 2009).

pH and Conductivity Measurement

The pH of the NEs was directly measured by a portable pH meter (Hanna Edge, USA) at 25 ± 2 °C. The electrical conductivity was determined at 25 ± 2 °C using the same device.

Preliminary Stability Tests

The preliminary stability of the NEs were evaluated by centrifugation, thermal stress and heating-cooling cycle tests. The NEs were analysed after centrifugation and thermal stress tests by visual observation regarding any physical instability issues. Afterwards, the stable NEs were taken for the heating-cooling cycle. These tests were carried out to predict the stable NEs in a short time before performing the long-term physical stability tests. Firstly, centrifugation was applied to 10 g of sample at 3500 rpm for 30 min with a laboratory centrifuge (ThermoScientific, USA) to accelerate possible instability issues (Altuntaş & Yener, 2015). Subsequently, thermal stress test was applied to the NEs that passed the centrifugation test successfully. Glass tubes containing 10 g of sample were submitted to a range of temperatures (from 40 up to 80°C, increasing by 5°C intervals) for 30 min at each temperature point using a thermostatic water-bath (Witeg WSB-30, Germany) (Altuntaş & Yener, 2015). A heating-cooling cycle test was performed to evaluate the NEs' stability at extreme temperature changes. For heating-cooling cycle test, the selected NEs were subjected to six cycles of heating and cooling. The samples were kept at 4°C for 48 hours and then at 40°C for 48 hours. The stability of the NEs as a result of the heating-cooling cycle was evaluated by visual observation, particle size analysis and zeta potential measurement (Shakeel et al., 2009). Only the NEs which represented no instability problems were selected for 3 months of physical stability tests.

Physical Stability Test

To investigate the physical stability of the selected NEs, the freshly prepared samples were stored at closed glass vials and placed at different temperatures for a period of 3 months: 25 ± 2 °C and 60% RH, 40 ± 2 °C and 75% RH, and 5 ± 3 °C. Samples were characterized with respect to their particle size, PDI, zeta potential, viscosity, pH and conductivity at predetermined time intervals. All experiments were performed in triplicate.

Microbiological Test

The antimicrobial effect of the selected NE formulation (F6P2) on yeasts was determined by the microdilution method reported by the Clinical Laboratory Standards Institute (CLSI) (Gong et al., 2019). As the yeast to be tested, *C. albicans* ATCC 10231 standard strain and four *C.albicans* strains from skin samples from Group Florence Nightingale Hospitals microbiology laboratories were used.

In this study, to determine the Minimum Inhibitor Concentration (MIC) value of test product against *C.albicans* strains, inoculums (0.5 - 2.5 x 10^3 cfu/ml) were prepared from fresh 24-48 hour cultures of standard and clinical *C.albicans* strains in MOPS buffered RPMI-1640 (Sigma) medium. After adding yeast suspensions to the test and market products, which were serially diluted in a 96-well U-bottom microplate, the microplates were incubated at 35°C for 48 hours. At the end of the incubation period, the lowest concentration without visible turbidity, which prevents the growth of *C.albicans* strains, was evaluated as MIC. In order to carry out a standardized study, the MIC values of fluconazole against the tested strains were also determined and these values were found to be within the quality control limits reported by CLSI.

In Vivo Studies

Study Design

In order to test the *in vivo* effectiveness of the selected NE formulation, the single-blind *in vivo* analyzes, compliance with the Helsinki Declaration, has been conducted after approval by the ethics committee of the Turkish Medicines and Medical Devices Agency affiliated to the Turkish Ministry of Health (Decision number: 58307721-512.99-44027). The study was initiated after 5 healthy volunteers aged 25-65 signed the written informed consent form. The selected volunteers applied the NE formulation as a thin layer to the SD area twice a day for 4 weeks. Inclusion and exclusion criteria for volunteers were determined as shown in the Table 2.

Table 2. The criteria for inclusion and exclusion of volunteers in the *in vivo* study.

The inclusion criteria	Persons must be male and female volunteers between the ages of 18 and 65 who have been diagnosed with SD.
	People should not have any other disease other than SD on their skin.
	Individuals must agree to sign a written informed consent form.
	No other product should be used at least seven days before the measurements.
	Volunteers must be available at the measurement times during the study.
	A different product should not be applied to the application area during the study period.
The exclusion criteria	Pregnant and lactating women.
	People using topical medication at the application site for any reason within the same period.
	Persons with known hypersensitivity to an ingredient in the formulation
	Persons with major hepatic and renal impairment.
	Persons involved in other clinical trials.
	Persons unable to adapt to work.
	Persons with hypersensitivity in the application area.

Dermatological Test (Skin Irritation Test)

In order to determine whether the formulation had any adverse effects on the skin of the volunteers, 0.2 grams of the test product was placed on the patch test material (IQ chamber) with an area of 1 cm^2, in contact with the forearm area, before the product was started to be applied. The volunteer was instructed to avoid contact with water and direct sunlight during the 48 hour observation period. At the end of the 48 hour period, the patch test material was removed and the volunteers' skin was checked for the presence of a skin reaction such as itching, redness, irritation, and edema (Altuntaş & Yener, 2015).

Preparation of The Measurement Environment

While taking measurements from the application areas of the volunteers based on the non-invasive biophysical methods, controlling the humidity and temperature of the measurement environment and preventing the skin of the volunteers from being exposed to direct sunlight are pivotal factors for the accuracy and reproducibility of the measurement results (Jansen van Rensburg, Franken, & Du Plessis, 2019). Therefore, the volunteers were tested after a 20-minute rest period in an air-conditioned room at 20 °C ± 2 temperature and 40 - 60% relative humidity in terms of dermal parameters such as skin pH, sebum, moisture and erythema.

Non-Invasive Biophysical Tests

Skin pH Measurement

To determine the effect of the test formulation on the skin pH of the volunteers, the pH of the skin was measured using the Skin-pH-Meter 900® (Courage & Khazaka, Electronic GmbH, Germany) (Altuntaş & Yener, 2015). At the beginning of the measurements,

the pH-meter was cleaned with distilled water. The probe tip was placed perpendicular to the skin and held on the skin for 3 seconds. After 3 seconds, the pH value was recorded. 5 different measurements were taken from the application area and the mean value were calculated.

Skin Sebum Measurement

The effect of the test formulation on the sebum secretion of the skin was determined by measuring with Sebumeter® SM 815 (Courage & Khazaka, Electronic GmbH, Germany) (Altuntaş & Yener, 2015). The cartridge was pressed lightly on the measuring device until the measuring time on the screen started counting down. When the countdown started, the cartridge was removed from the device and the measurement was started. During the 30 second time, the cartridge was held vertically in the measuring area on the skin. At the end of the period, the cartridge was re-inserted into the device and the measured value on the screen was recorded. 3 different measurements were taken from the application area and the mean value were calculated.

Skin Moisture Measurement

In order to determine the moisturizing effect of the test formulation on the skin, the water content of the skin was measured by Corneometer® CM 825 device (Courage & Khazaka, Electronic GmbH, Germany), which is based on the principle of electrical capacitance method. The water content value on the screen was recorded 1 second after the probe of the device came into contact with the skin. The results are given in arbitrary units (AU), where 1 unit of AU is estimated to correspond to 0.2 - 0.9 mg of water per gram of stratum corneum (De Melo & Maia Campos, 2018). 5 different measurements were taken from the application area and the mean value were calculated.

Skin Erythema Measurement

The pathological parameter, erythema (hemoglobin content), was evaluated photometrically with a Mexameter® 18 (Courage and Khazaka Electronic GmbH, Cologne, Germany) according to the principle of remission (Yilmaz & Borchert, 2006). The erythema value on the screen was recorded 1 second after the probe of the device came into contact with the skin. 3 different measurements were taken from the application area and the mean value were calculated.

Subjective Evaluation

After the end of the *in vivo* test period, the volunteers were also subjected to a subjective evaluation regarding the sensory and visual effects of the test formulation on skin. The volunteers were asked to score their preferences from 0 to 5 (dissatisfaction – most satisfaction) for the first 10 questions and for the last question, yes/no answers were received from the volunteers according to questionnaires specifically designed for the experiment.

Statistical Analysis

The *in vitro* data are expressed as means ± standard deviation (SD) (n = 3). The tests used for the statistical analysis of the results from the human study depended on the type of sampling distribution found, verified by tests of normality (Shapiro-Wilk test, Histogram, QQ-plot and box plot) of distribution and homogeneity of the variances involved in the experiment. Statistical differences were determined by the software package SPSS for Windows, Version 21 (SPSS Inc., Chicago, IL, USA)., using the Wilcoxon test to analyze the results from the human study. *P*-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Preparation and Characterization of Nanoemulsions

In our study, NEs containing oily phase with 3 different concentrations (%5, %6.68, %10 (w/w)) were successfully manufactured by ultrasonic emul-

sification method. Two different surfactant and cosurfactant mixtures (Poloxamer 188/Transcutol® HP and Cremophor® A25/Transcutol® HP) with 1:1 (w/w) and 1:2 (w/w) surfactant and cosurfactant ratios were tried. Poloxamer 188 (5% w/w) and Transcutol® HP (10% w/w) was the only surfactant and cosurfactant combination that resulted in a stable formulation and therefore was chosen for further study. The selected NEs were then characterized and the stability of them was studied. According to the results of the stability tests, among the tested NE formulations, the NE formulation with the least change in terms of particle size, PDI and, zeta potential values was selected as the optimum NE formulation.

There are various methods to making NEs, but these are usually inconvenient or costly and a phase diagram needs to be prepared to obtain a stable emulsion. However, NEs prepared with polymeric surfactants can be stably produced with small droplet sizes without a phase diagram (Wik, Bansal, Assmuth, Rosling, & Rosenholm, 2020). In this study, Poloxamer 188, a triblock copolymer, was chosen as surfactant to stabilize the NEs due to its well-known stabilization property and FDA approval status for human use. This polymeric surfactant has already been used in numerous studies to make a stable NE formulation (Wulff-Pérez, Gálvez-Ruíz, De Vicente, & Martín-Rodríguez, 2010; Wulff-Pérez, Torcello-Gómez, Gálvez-Ruíz, & Martín-Rodríguez, 2009). Transcutol®, a potent, non-toxic, biodegradable solvent, was also included as a co-surfactant to obtain a steady liquid interfacial film. Similar to our study, there are various researches in the literature using Transcutol® as a co-surfactant in their formulations (Goyal, Arora, & Aggarwal, 2012; Solanki, Sarkar, & Dhanwani, 2012).

As a result, all of the prepared formulations had low viscosity, and exhibited a milky and bluish appearance. Characterization study revealed that the average droplet diameter, PDI and zeta potential of all NEs were varied from 102.70 ± 3.10 nm to 126.30 ± 0.90 nm, 0.147 ± 0.01 to 0.253 ± 0.01 and - 21.40 ± 1.45 mV to - 34.10 ± 0.6 mV, respectively. In the prepared NEs, the droplet sizes of less than 200 nm of the dispersed oil phase, the PDI values of less than 0.3, and zeta potential values (ζ) of less than −20 mV, indicate their high kinetic stability and they are considered to be convenient for topical applications (Kumar, & Mandal, 2018; Pongsumpun, Iwamoto, & Siripatrawan, 2020).

Preliminary Stability Tests

NEs are thermodynamically unstable colloidal disperse systems, and thus stability issues such as creaming, sedimentation, coalescence or phase separation are likely to arise on long-term storage (Wik, Bansal, Assmuth, Rosling, & Rosenholm, 2020). Therefore, accelerated stability tests (centrifugation and thermal tests) were performed on NEs prepared with Poloxamer 188 and Transcutol® HP to accelerate emulsion degradation. The test result was recorded by observing any signs of physical stability issues. As a result of the centrifuge test, it was observed that all the tested formulations remained stable, indicating that the NEs were durable to centrifugal forces. However, thermal stress caused stability problems such as coalescence and creaming, which are frequently seen in colloidal systems in formulations prepared with 10% Poloxamer 188 and 10% Transcutol® HP in this study. On the other hand, it was determined that only the NEs containing 5% Poloxamer 188 and 10% Transcutol® HP remained stable after thermal stress test.

The heating/cooling cycle (H-C) test might be useful to evaluate the NEs stability at extreme temperature changes (Mota Ferreira et al., 2016). In our study, we observed that the NEs did not show any signs of instability issues after six H-C cycles. When the results were investigated, it was found that the droplet sizes of the NEs maintained at the desired nano-size range (min: 105.80 ± 0.95 nm and max: 140.70 ± 3.30)

after six H-C cycles. While the average droplet diameter of the selected NE formulation (F6P2) was 108.4 ± 0.90 nm before the test, it was found to be 128.00 ± 2.30 nm with a slight increase after the test (Figure 1). In addition, PDI values of the NEs remained under 0.3 after six H-C cycles. So, it can be concluded that all NEs tested had a similar droplet size distribution even under extreme conditions.

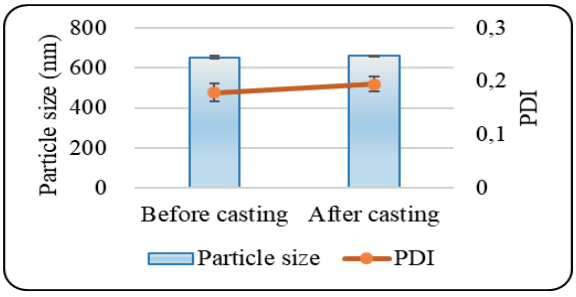

Figure 1. Comparison of average droplet size and PDI value of the selected NE formulation (F6P2) before and after the H–C test (means ± SD, n = 3).

Physical Stability Test

In order for the formulation to remain stable throughout its shelf life, an appropriate production process and formulation excipients should be selected, but the formulation should also be resistant to environmental conditions such as heat, humidity or light. To test the stability of the NEs, physical stability studies were conducted on selected NEs over a 3-month period in three different stability test environments. Organoleptic control, average droplet diameter, PDI, zeta potential, viscosity, pH and conductivity were monitored by taking measurements at pre-determined time points during the storage period.

All selected NE formulations in physical stability study remained in a homogenous state and showed no physical or phase changes after 3 months under all storage temperatures. According to Table 3, the droplet size, PDI, and zeta potential value of the selected NE did not change statistically over three months in three different storage environments. The droplet size of the selected NE was maintained between 120.90 and 129.80 nm for 3 months. All selected NE have low PDI values (below 0.2) during the 3-month test period, indicating a narrow droplet size distribution and superior stability of the system.The zeta potential of the selected NE scarcely changed in the range of -20.50 to -29.20 mV during the 3 months of the physical stability study. This indicated that the different storage temperatures did not have a significant effect on the repulsive forces between the droplets of the NEs.

If the pH values of the NEs measured at different time points are in the skin pH range (4 - 6), it can be stated that the formulations can be used safely on human skin (Muhammad, Akhtar, Haji, Mustafa, & Murtaza, 2014). In our study, it can be clearly seen that the change in pH of the selected NEs is negligible at different measurement points under different storage conditions and they are suitable for topical application. Conductivity measurement is a useful technique for determining emulsion type and observing changes during production or storage (Kildaci, Budama-Kilinc, Kecel-Gunduz, & Altuntas, 2021). Accordingly, the conductivity measurement results showed that there were no significant electrical conductivity changes in any of the NEs in three different stability environments during the 3 months. Korhonen et al. reported that oil-in-water emulsions have higher conductivity values compared to water-in-oil emulsions, since the outer phase is water (Korhonen, Niskanen, Kiesvaara, & Yliruusi, 2000). In line with this information, high conductivity values of the NEs produced in our study indicate the external phase of the NEs is aqueous.

Table 3. Physical stability test results of the selected NE formulation (means ± SD, n = 3).

Formulation	Temp.	Time	Average Droplet Size (nm)	Polydispersity Index (PDI)	Zeta potential (mV)	pH	Conductivity (µs/cm)
F6P2	5 °C	T30	128.90 ± 2.10	0.166 ± 0.01	-20.50 ± 0.70	4.57 ± 0.01	80.00 ± 1.24
		T60	127.40 ± 1.60	0.149 ± 0.01	-23.80 ± 1.00	4.52 ± 0.03	85.40 ± 2.57
		T90	123.10 ± 2.10	0.165 ± 0.04	-27.40 ± 0.90	4.51 ± 0.03	86.30 ± 3.44
	25 °C	T1	126.30 ± 0.90	0.147 ± 0.01	-21.40 ± 1.45	4.58 ± 0.02	74.20 ± 2.86
		T30	129.80 ± 1.00	0.159 ± 0.01	-23.30 ± 0.60	4.52 ± 0.01	93.80 ± 1.05
		T60	125.50 ± 2.30	0.160 ± 0.04	-26.40 ± 3.10	4.56 ± 0.01	114.60 ± 3.62
		T90	123.60 ± 0.50	0.133 ± 0.10	-27.20 ± 0.70	4.52 ± 0.02	129.50 ± 4.23
	40 °C	T30	125.90 ± 2.40	0.165 ± 0.017	-22.00 ± 0.90	4.52 ± 0.02	107.20 ± 2.21
		T60	120.90 ±0.70	0.165 ± 0.021	-29.20 ± 0.50	4.53 ± 0.03	152.30 ± 0.96
		T90	125.00 ± 2.80	0.159 ± 0.058	-25.80 ± 0.60	4.61 ± 0.01	157.30 ± 3.25

The results obtained from the rheological study for the selected NE formulation is represented in Table 4. It can be observed that the NE presented Newtonian behavior characterized by the independence of viscosity from the applied shear rate. Such rheological feature was observed in all NE formulations in this study. Our experiments are consistent with previous results (Shakeel, 2017; Erramreddy, Tu, & Ghosh, 2017).

Table 4. Viscosity measurements of the selected NE during the 3 months of the physical stability study at 5 ± 3 °C; 25 ± 2 °C and 60% RH; 40 ± 2 °C and 75% RH.

Speed (rpm)	Viscosity (cP)									
	T1	T30			T60			T90		
	25 °C	4 °C	25 °C	40 °C	4 °C	25 °C	40 °C	4 °C	25 °C	40 °C
70	6.63	7.01	6.45	6.76	6.46	6.64	6.74	6.45	6.68	6.98
75	6.62	6.71	6.38	6.35	6.46	6.79	6.02	8.11	6.54	7.15
80	6.45	6.54	6.25	6.37	6.36	6.76	5.97	7.85	6.21	7.03
85	6.39	6.46	6.26	6.29	6.32	6.8	5.92	7.54	6.08	6.85
90	6.39	6.25	6.18	6.31	6.21	6.77	5.89	7.34	6.10	6.69
95	6.47	6.26	6.18	6.25	6.20	6.74	5.92	7.30	5.92	6.68
100	6.42	6.29	6.10	6.26	6.12	6.71	5.89	7.06	5.89	6.67
105	6.42	6.18	6.18	6.28	6.10	5.69	5.85	7.10	5.85	6.66
110	6.43	6.18	6.06	6.23	6.00	6.67	5.89	7.08	5.83	6.60
115	6.43	6.14	6.06	6.26	5.96	6.71	5.86	6.99	5.52	6.65

Microbiological Test

The selected NE formulation was found to be effective on *C.albicans* in microbiological test. The MIC values of the tested formulation are as shown in Table 5. According to these results, the selected formulation F6P2 has showed good antifungal activity against *C. albicans*, especially clinical strains. *C. albicans* is an important pathogen that is representative for yeast fungi in many antifungal studies in the literature, and the antifungal activity obtained from that studies, indicates that the studied substance might be effective against many yeasts, including *M. furfur*. This efficacy was also similar to the antifungal activities of the active essential oils' in the NE formulation.

Table 5. *In vitro* susceptibility of *C.albicans* and its clinical isolates to the selected NE formulation by microdilution method.

Yeasts	MIC values (%)
	Test formula (F_6P_2)
***C.albicans* ATCC 10231**	6.75
Clinical isolate 1	1.6
Clinical isolate 2	0.4
Clinical isolate 3	0.8
Clinical isolate 4	3.1

***In Vivo* Studies**

The tested NE formulation did not cause any visual skin irritation after the skin irritation patch test at the end of the 48 hour period. Thus, it can be concluded that the tested formulation was well tolerated and compliance was achieved.

Non-invasive biophysical techniques are more preferred by consumers and cosmetic researchers, are more ethically acceptable and have great advantages such as reproducibility. They enable the detection of invisible changes in the skin or hair without causing pain and discomfort. They are also more suitable for statistical evaluation (Altuntaş & Yener, 2015). Therefore, we performed a single-blind *in vivo* assay to test the safety and efficacy of the selected NE formulation in SD patients using non-invasive biophysical techniques.

Some external factors such as soap, detergent and cosmetics can negatively change the normal pH of the skin. Changes in skin pH can cause irritation or inhibition of the keratinization process (Altuntaş & Yener, 2015). In order to investigate the effect of the formulation on skin pH, pH measurements were taken from the application sites of the volunteers before the test and after the 4-week product application period. Consequently, there was no significant difference between the skin pH index, before and 4 weeks after the application with the tested NE formulation ($p > 0.05$) (Table 6).

Suchonwanit et al. conducted a study to evaluate the biophysical and physiological profiles in scalp SD (Suchonwanit, Triyangkulsri, Ploydaeng, & Leerunyakul, 2019). As a result, they showed that the mean skin moisture content was significantly lower in SD patients compared to healthy volunteers ($p < 0.05$). Skin surface lipid was also measured significantly higher in SD group ($p < 0.05$). In line with this study, we detected low moisture and high sebum contents in the skin of patients with SD at the beginning of the study. While the mean skin hydration index significantly increased after the tested water based formula was applied (17.12 versus 27.04 AU; $p = 0.001$), the mean skin sebum content significantly decreased (25.80 versus 18.90 mg/cm^2; $p = 0.008$) (Table 6). The possible explanation for the increase in moisture content in the application area can be made as the changing epidermal barrier function of the skin resulting from the inflammatory process is restored to its healthy state with the emollients and humectants included in the formulation composition.

The increase in the skin moisture content was accompanied by a decrease in skin erythema. After 4 weeks of application of the test formulation, there was a statistically significant reduction of the mean skin erythema index compared to baseline (T0) (585.60 versus 567.30; $p = 0.001$) (Table 6).

Table 6. *In vivo* study variables results before and after treatment with the selected NE formulation.

Variable	Before administration (mean)	Four weeks after administration (mean)	*p* value
Skin pH	5.70 ± 0.2	5.60 ± 0.1	0.20
Skin sebum content (mg/cm^2)	25.80 ± 19.0	18.90 ± 12.2	0.008
Skin hydration index (AU)*	17.12 ± 17.1	27.04 ± 19.3	0.001
Skin erythema index (AU)*	585.60 ± 40.5	567.30 ± 34.0	0.001

*AU, arbitrary units

Also, when patients were asked to state their opinions about the test formulation, they agreed that the product was 80% effective in reducing skin redness, 84% in reducing dandruff, 72% in reducing skin itching, and 64% in reducing skin oiliness. 72% of the volunteers expressed their satisfaction with the test product they used.

Table 7: Questionnaire scores given by the volunteers (n = 5).

Evaluation criteria	The questions in the questionnaire	Score	percentage (%)
0 to 5 (dissatisfaction – most satisfaction)	Did the NE serum you used reduce the redness on your skin?	4.0	80
	Did the NE serum you used reduce the amount of dandruff on your skin?	4.2	84
	Did the NE serum you used reduce itching?	3.6	72
	Did the NE serum you used relief your skin?	3.4	68
	Did the NE serum you used moisturize your skin?	3.6	72
	Did the NE serum you used reduce the oiliness on your skin?	3.2	64
	Is the NE serum you used easily absorbed by your skin?	4.8	96
	Did the NE serum you used give your skin a soft feeling?	3.2	64
	Did the NE serum you used cause any allergic reaction such as redness, itching, burning on your skin?	0.2	4
	Are you satisfied with the NE serum you used?	3.8	76
yes/no	Would you consider using the NE serum again?	4 - yes 1- no	80

CONCLUSION

In this study, a herbal-based NE formulation was successfully developed against SD. For this purpose, a formulation in NE form with moisturizing, regenerating and healing effects was prepared in a stable form using constituents known to have both antifungal and antimicrobial activity (tea tree, sage, cinnamon and oregano essential oils, and honey) together with other antioxidant substances (extracts from *Aloe vera,* colloidal oatmeal, and liquorice; grape seed oil and sesame oil).

This formulation was subjected to an *in vitro* microbiological study and *in vivo* non-invasive instrumental analyzes and a subjective questionnaire test to demonstrate its therapeutic potential. When all findings were evaluated, the herbal based NE formulation was considered to be effective significantly against SD. Since SD is an incurable disease, this study will thought be a good reference for ongoing research on the subject.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Developing hypothesis (F.G.Y., E.A.), experimenting (F.D., E.A., S.D.), preparing the study text (E.A.), reviewing the text (F.G.Y., E.A.), statistics, analysis and interpretation of the data (F.D., E.A.), literature research (F.D., E.A.).

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RESEARCH ARTICLE

Synthesis and Antibacterial Evaluation of Novel Benzimidazole, Benzothiazole, Benzofurane, and Naphtofurane Derivatives of Aminothiazoles

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Synthesis and Antibacterial Evaluation of Novel Benzimidazole, Benzothiazole, Benzofurane, and Naphtofurane Derivatives of Aminothiazoless

SUMMARY

The thiazole ring is the core of bioactive molecules that generate broad activity. These activities include anticonvulsant, antimicrobial, antituberculosis, antiviral, etc. In this work, starting from seconder/cyclic amines, new compounds containing thiazole and benzimidazole/benzothiazole/benzofurane/naphtofurane rings were synthesized, and their antimicrobial effects were evaluated. 9 compounds were synthesized by converting the seconder and cyclic amines to thiourea, and continued by thiazole ring closure. Ring closure was achieved by methylene-carbonyl condensation except conventional methods. Compound characterization was realized by FT-IR, ^{1}H NMR and ^{13}C NMR and HRMS. Compounds did not show significant activity on bacterial strains. Nine aminothiazole derivatives have been synthesized successfully. Compounds did not show important antibacterial activity and thus were evaluated as inactive.

Key Words: *antibacterial, aminothiazole, benzothiazole, benzofurane, naphtofurane*

Aminotiyazollerin Benzimidazol, Benzotiyazol, Benzofuran ve Naftofuran Türevlerinden Yeni Bileşiklerin Sentezi Ve Antimikrobiyal Etkilerinin Değerlendirilmesi

ÖZ

Tiyazol halkası, birçok alanda biyolojik aktivite oluşturan moleküllerin çekirdeğidir. Bu aktiviteler arasında antikonvülsan, antimikrobiyal, antitüberküloz, antiviral vb. farmakolojik etkiler yer almaktadır. Bu çalışmada sekonder/siklik aminlerden yola çıkılarak tiyazol ve benzimidazol/benzotiyazol/benzofuran/naftofuran halkaları içeren yeni tiyazol türevleri sentezlenmiş ve antimikrobiyal etkileri değerlendirilmiştir. Bileşiklerin sentezinde, sekonder veya siklik aminler tiyoüreye dönüştürülerek 9 bileşik sentezlenmiş ve tiyazol halka kapanması ile devam edilmiştir. Halka kapatma, konvansiyonel yöntemler dışında metilen-karbonil kondenzassyonuyla gerçekleşmiştir. Bileşiklerin karakterizasyonu FT-IR, ^{1}H NMR ve ^{13}C NMR ve HRMS ile gerçekleştirilmiştir. Bileşikler, bakteri suşları üzerinde önemli aktivite göstermedi. 9 aminotiyazol türevi başarıyla sentezlenmiştir. Bileşikler önemli bir antibakteriyel etki göstermediğinden inaktif olarak tanımlanmıştır.

Anahtar Kelimeler: *antibakteriyel, aminotiyazol, benzotiyazol, benzofuran, naftofuran*

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INTRODUCTION

The discovery and clinical use of antibacterial drugs are one of the most outstanding achievements in the history of health (Gajdacs, 2019). Human lifespan has increased since the 1850s, which was manifested by the improvements that treated or prevented infectious diseases (Hayward, 2016). Today, however, bacterial resistance is growing with the decrease in the development of antibacterial drugs, and as a result, treatment options become more limited (Nambiar, 2014). Over the past few years, there has been increasing focus on the challenge of antimicrobial resistance. Despite discovering of new developments, there is still a long way to go to eliminate certain threats (Tomasi, 2018).

Individual infectious diseases usually have their specific bacterial strains. Among the microbial strains, *E. coli*, which causes diseases such as urinary tract infections, sepsis, meningitis, enteric, diarrhoeal (Kaper, 2004); *S. aureus*, which causes diseases such as bacteremia, infective endocarditis, pneumonia (Dayan, 2016; Oliveira, 2018); *Salmonella* species causes gastroenteritis, septicemia, and fever (Jajere, 2019).

Thiazole is the core of compounds with a wide range of activities (Rouf, 2015; Chhabria, 2016; Kashyap, 2018; Kumawat, 2018). These activities include anticonvulsant, antimicrobial, antituberculosis, antiviral, etc. (Parekh, 2012; Ayati, 2015; Althagafi, 2019; Singh, 2020; Borcea, 2021; Petrou, 2021). Previous research revealed that thiazole derivatives will be an essential basis for producing biologically active compounds. Also, the activity of compounds with a 2-aminothiazole nucleus is present (Makam, 2014; Wan, 2020; Elsadek, 2021). Besides, bicyclic heterocyclic rings are highly interested in drug discovery, with their potential of making hydrophobic and hydrogen bonding interactions. Benzimidazole, benzofurane, benzothiazole, and naphtofurane are among these rings, and they are present in drugs e.g., albendazole, pramipexole, amiodarone, and rubicordifolin, respectively. Conventional thiazole synthesis is realized by the Hantzsch method. However, there are also different ring synthesis methods. In one, benzoyl thioureas are reacting with bromoacetyl arenes, which include two mechanistic steps; the first, include the attack of sulfur to acyl bromide, and the second represents the carbonyl-methylene condensation (Ried, 1976; Sabbaghan, 2011; Belveren, 2017). Also, our group previously synthesized a series of compounds with this type of thiazole closure, and reported their acetylcholinesterase inhibitory activity (Sahin, 2018; Demirayak, 2019). In this study, cyclic and acyclic amines are converted to thiourea, then reacted with acyl bromides to obtain nine novel compounds. These compounds structures were elucidated and antibacterial activity was tested. A significant number of targets exist for antibacterial activity, including DNA gyrases, cell membrane disruption, protein synthesis, nucleic acid synthesis, biological metabolic compound synthesis inhibitors, secA inhibitors, etc. Screening of novel compounds such as in this study is essential to find their activity and then investigate their mechanism of action. However, as no significant activity is observed in the compounds (1-9), the mechanism of action is not investigated (Chen, 2010; Silver, 2016).

MATERIAL AND METHODS

Chemistry

Starting and intermediate compounds were supplied from Sigma-Aldrich, VWR, or Honeywell. Melting points (m.p.) were determined by Stuart MP90 digital melting point apparatus. Synthesis steps were checked by thin-layer chromatography (TLC) using Silica Gel 60 F254 TLC plates. Spectroscopy was measured with the following instruments: Fourier transform-infrared spectroscopy (FT-IR) (Perkin Elmer S, Shimadzu Affinity 1S spectrophotometer (Shimadzu, Tokyo, Japan); Nuclear magnetic resonance (NMR), Agilent 300 MHz NMR spectrometer (Agilent Technologies, California, USA), in dimethyl sulfoxide (DMSO)-d6. TMS was used as a standard. For high resolution mass spectroscopy, M+1 peaks were determined by Shimadzu 8040 Liquid Chromatography with tandem mass spectrometry (LC/MS/MS) system

(Shimadzu, Tokyo, Japan).

Bromination of 2-acetylbenzimidazole, 2-acetylbenzofurane and 2-acetylnaphtofurane

Heterocyclic methyl ketones were dissolved in acetic acid. An equal mole of bromine was diluted in AcOH, added dropwise, and refluxed until the reaction was complete. Then reaction left to cool, poured into cold water, and precipitation was collected, and recrystallized from ethanol. Checked for their melting point and compared with literature data.

Bromination of 2-acetylbenzothiazole

Heterocyclic methyl ketones were dissolved in ethyl acetate. An equal mole of copper (II) bromide ($CuBr_2$) were diluted in ethyl acetate, added dropwise, and refluxed until the reaction was complete. Then reaction left to cool, filtered off, and the liquid was evaporated. Obtained solid materials were recrystallized from ethanol. Checked for their melting point and compared with literature data.

Synthesis of benzoylthioureas

Amines (dimethylamine, pyrrolidine, piperidine, hexamethyleneimine, and morpholine) were treated with NH_4SCN and benzoyl chloride to obtain benzoylthioureas (Figure 1). Firstly, ammonium thiocyanate was dissolved in acetone. Benzoyl chloride (diluted in acetone) was added dropwise at room temperature. Then the equal mole of the corresponding amine is added and mixed for 10 min at room temperature and 1 h reflux. Washed with warm ethanol and used as it is for the next step.

Synthesis of tested compounds 1-9

Benzoylthioureas and bromoacetyl aryl derivatives were refluxed in ethanol to get final compounds 1-9 by a methylene-carbonyl condensation (Demirayak, 2019), as shown below (Figure 1). After the reaction was complete, mixture was left to cool, and precipitation was collected, and recrystallized from ethanol. Compound characterization has been made by IR, ^{1}HNMR, ^{13}CNMR, and HRMS spectra.

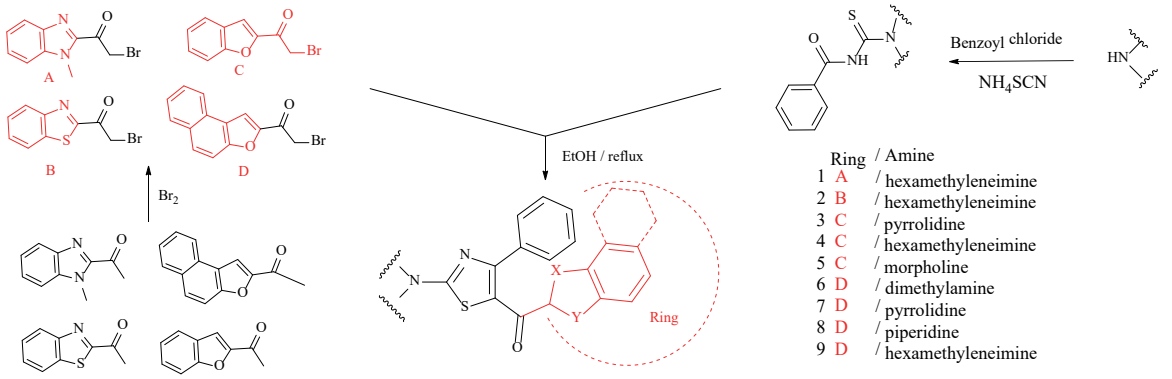


Figure 1. General synthesis of compounds

(2-(azepan-1-yl)-4-phenylthiazol-5-yl)(1-methyl-1H-benzo[d]imidazol-2-yl)methanone (Compound 1)

Yield 72%, light yellow, mp 230.3 °C. FT-IR ῡmax (cm^{-1}): 3041 to 2857 (C—H), 1620 (C═O), 1539 to 1330 (C═C, C═N). ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ: 1.56 (4H, brs, hexamethylene-CH_2), 1.80 (4H, brs, hexamethylene-CH_2), 3.69 (4H, brs, N(CH_2)$_2$), 3.96 (3H, s, N-CH_3), 7.25-7.67 (9H, m, Ar-H). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ: 27.47, 32.23 (Benzimidazole-CH_3), 111.57, 121.01, 123.49, 125.19, 127.78, 129.00, 129.81, 136.57, 136.72, 141.10, 148.22, 152.37, 164.13, 173.22 (C=O). HRMS (m/z): $[M+H]^+$ calcd for $C_{24}H_{24}N_4OS$: 416.1784; found: 416.1786.

(2-(azepan-1-yl)-4-phenylthiazol-5-yl)(benzo[d]thiazol-2-yl)methanone (Compound 2)

Yield 57%, light yellow solid, mp 176.1 °C. FT-IR ῡmax (cm^{-1}): 3050 to 2848 (C—H), 1602 (C═O), 1520 to 1280 (C═C, C═N). ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ: 1.58 (4H, brs, CH_2), 1.83 (4H,

brs, CH_2), 3.75 (4H, brs, $N(CH_2)_2$), 7.23-7.50 (3H, m, Ar-H), 7.51-7.80 (4H, m, Ar-H), 8.06-8.35 (2H, m, Ar-H). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ: 27.49($(CH_2)_3$), 54.55 ($N(CH_2)_2$), 123.42, 125.15, 127.66, 127.91, 129.43, 130.05, 136.24, 142.50, 144.29, 153.53, 169.92 (C=O). HRMS (m/z): $[M+H]^+$ calcd for $C_{23}H_{21}N_3OS_2$: 420.1199; found: 420.1199.

Benzofuran-2-yl(4-phenyl-2-(pyrrolidin-1-yl) thiazol-5-yl)methanone (Compound 3)

Yield 65.5%, orange solid, mp 166.8 °C. FT-IR ῡmax (cm^{-1}): 3108 to 2881 (C─H), 1542 (C═O), 1459 to 1299 (C═C, C═N). ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ: 2.02 (4H, t, J: 6.66 Hz, pyrrolidine-CH_2), 3.51 (4H, brs, $N(CH_2)_2$), 7.15-7.28 (4H, m, Ar-H), 7.30-7.39 (2H, m, Ar-H), 7.42-7.50 (2H, m, Ar-H), 7.53-7.60 (1H, m, Ar), 7.64 (1H, d, J: 7.72 Hz, Ar). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ: 25.67 ($(CH_2)_2$), 50.15 ($N(CH_2)_2$) 112.11, 113.38, 123.39, 124.20, 127.09, 127.94, 128.10, 128.36, 128.45, 129.07, 129.50, 131.99, 136.35, 152.47, 154.63, 161.41, 168.27, 174.59 (C=O). HRMS (m/z): $[M+H]^+$ calcd for $C_{22}H_{18}N_2O_2S$: 375.1162; found: 375.1167.

(2-(azepan-1-yl)-4-phenylthiazol-5-yl)(benzofuran-2-yl)methanone (Compound 4)

Yield 67.7%, yellow solid, mp 152.8 °C. FT-IR ῡmax (cm^{-1}): 3059 to 2925 (C─H), 1538 (C═O), 1464 to 1296 (C═C, C═N). ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ: 1.57 (4H, brs, hexamethylene-CH_2), 1.81 (4H, brs, hexamethylene-CH_2), 3.68 (4H, brs, $N(CH_2)_2$), 7.15-7.83 (8H, m, Ar), 7.89 (1H, d, J: 7.71 Hz, Ar), 8.15 (1H, d, J: 0.74 Hz, Ar). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ: 25.23, 27.47, 111.39, 111.96, 112.11, 112.82, 113.42, 115.12, 118.80, 122.91, 123.40, 124.20, 124.73, 127.94, 128.11, 129.09, 129.49, 136.30, 142.02, 179.17 (C=O). HRMS (m/z): $[M+H]^+$ calcd for $C_{24}H_{22}N_2O_2S$: 403.1475; found: 403.1480.

Benzofuran-2-yl(2-morpholino-4-phenylthiazol-5-yl)methanone (Compound 5)

Yield 69.8%, brown solid, mp 199.3 °C. FT-IR ῡmax (cm^{-1}): 3014 to 2870 (C─H), 1593 (C═O), 1519 to 1329 (C═C, C═N). ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ: 3.61 (4H, t, J:5.01 Hz, $N(CH_2)_2$), 3.75 (4H, t, J:5.76 Hz, $O(CH_2)_2$), 7.19-7.28 (4H, m, Ar-H), 7.33-7.49 (5H, m, Ar-H), 7.65 (1H, d, J:8.02 Hz, Ar-H). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ: 48.32($N(CH_2)_2$), 65.79($O(CH_2)_2$), 112.17, 114.01, 119.57, 123.53, 124.30, 127.05, 128.20, 129.20, 129.47, 136.0, 152.27, 154.75, 160.36, 175.0 (C=O). HRMS (m/z): $[M+H]^+$ calcd for $C_{22}H_{18}N_2O_3S$: 391.0507; found: 391.0508.

(2-(Dimethylamino)-4-phenylthiazol-5-yl) (naphtho[2,1-b]furan-2-yl)methanone (Compound 6)

Yield 71.3%, yellow solid, mp 181.7 °C. FT-IR ῡmax (cm^{-1}): 3055 to 2882 (C─H), 1583 (C═O), 1542 to 1302 (C═C, C═N). ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ: 3.20 (6H, s, $N(CH_3)_2$), 7.13-7.19 (3H, m, Ar-H), 7.39-7.55 (4H, m, Ar), 7.61-7.67 (1H, m, Ar-H), 7.90 (1H, d, J: 9.07 Hz, Ar-H), 8.01 (1H, d, J:7.76 Hz, Ar-H), 8.06 (1H, s, Ar-H), 8.26 (1H, d, J:8.03 Hz, Ar-H). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ: 41.5 ($N(CH_3)_2$), 112.72, 113.25, 113.28, 120.02, 122.87, 124.16, 125.91, 126.44, 127.21, 127.73, 127.93, 128.11, 129.01, 129.31, 129.48, 130.45, 131.02, 136.45, 151.96, 152.83, 160.66, 171.72, 174.36 (C=O). HRMS (m/z): $[M+H]^+$ calcd for $C_{24}H_{18}N_2O_2S$: 399.1162; found: 399.1158.

Naphtho[2,1-b]furan-2-yl(4-phenyl-2-(pyrrolidin-1-yl)thiazol-5-yl)methanone (Compound 7)

Yield 64.6%, brown solid, mp 186.3 °C. FT-IR ῡmax (cm^{-1}): 3054 to 2961 (C─H), 1541 (C═O), 1458 to 1311 (C═C, C═N). ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ: 2.02 (4H, brs, $(CH_2)_2$), 3.48 (4H, brs, $N(CH_2)_2$), 7.09-7.26 (3H, m, Ar-H), 7.40-7.74 (5H, m, Ar-H), 7.87 (1H, t, J:9.03 Hz, Ar-H), 7.98-8.02 (2H, m, Ar-H), 8.25 (1H, d, J: 8.04 Hz, Ar-H). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ: 25.68 ($(CH_2)_2$), 50.15 ($N(CH_2)_2$), 108.53, 112.17, 112.71, 113.10, 122.87, 124.14, 125.89, 128.09, 129.49, 130.45, 131.81, 136.54,

140.22, 152.03, 152.79, 160.93, 168.20, 174.28(C=O). HRMS (m/z): $[M+H]^+$ calcd for $C_{26}H_{20}N_2O_2S$: 425.1318; found: 425.1321.

Naphtho[2,1-b]furan-2-yl(4-phenyl-2-(piperidin-1-yl)thiazol-5-yl)methanone (Compound 8)

Yield 83.9%, brown solid, mp 169.1 °C. FT-IR ῡmax (cm^{-1}): 3106 to 2857 (C—H), 1573 (C═O), 1541 to 1336 (C═C, C═N). ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ: 1.64 (6H, brs, $(CH_2)_3$), 3.62 (4H, brs, $N(CH_2)_2$), 7.08-7.20 (3H, m, Ar-H), 7.39-7.58 (4H, m, Ar-H), 7.64 (1H, t, J: 7.05 Hz, Ar-H), 7.89 (1H, d, J: 9.11 Hz, Ar), 8.0 (1H, d, J:8.08 Hz, Ar-H), 8.08 (1H, brs, Ar-H), 8.26 (1H, d, J: 7.91 Hz, Ar-H). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ: 23.81, 25.26, 49.44($N(CH_2)_2$), 112.72, 113.36, 119.40, 122.89, 124.16, 125.91, 127.74, 127.94, 128.11, 129.03, 129.30, 129.46, 129.52, 130.46, 136.39, 151.98, 152.88, 160.50, 171.39, 174.42(C=O). HRMS (m/z): $[M+H]^+$ calcd for $C_{27}H_{22}N_2O_2S$: 439.1475; found: 439.1477.

(2-(Azepan-1-yl)-4-phenylthiazol-5-yl)(naphtho[2,1-b]furan-2-yl)methanone (Compound 9)

Yield 77.5%, yellow solid, mp 186.7 °C. FT-IR ῡmax (cm^{-1}): 3107 to 2877 (C—H), 1519 (C═O), 1475 to 1311 (C═C, C═N). ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ: 1.56 (4H, brs, hexamethylene-CH_2), 1.81 (4H, brs, $(CH_2)_2$), 3.68 (4H, brs, $N(CH_2)_2$), 7.07-7.22 (3H, m, Ar-H), 7.41 (1H, dd, J: 9.04 Hz, 0.60 Hz, Ar), 7.45-7.58 (3H, m, Ar-H), 7.64 (1H, t, J:7.05 Hz, Ar-H), 7.88 (1H, d, J: 9.08 Hz, Ar), 8.01 (1H, d, J: 9.03 Hz, Ar-H), 8.08 (1H, d, J: 0.74 Hz, Ar-H), 8.26 (1H, d, J:7.97 Hz, Ar-H). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ: 26.32, 27.49, 28.26, 51.20($N(CH_2)_2$), 112.70, 113.13, 119.33, 122.88, 124.16, 125.89, 127.71, 127.94, 128.08, 128.98, 129.29, 129.41, 129.50, 130.45, 136.50, 152.04, 152.79, 160.69, 170.98, 174.31(C=O). HRMS (m/z): $[M+H]^+$ calcd for $C_{28}H_{24}N_2O_2S$: 453.1631; found: 453.1649.

Antimicrobial activity

Minimum inhibitory concentration assay

Gram-positive and gram-negative bacterial strains such as *E. coli* (ATCC8739), *S. aureus* (ATCC6538), *Salmonella* sp. (ATCC700623) were incubated and compounds were tested on these microorganisms. A previous procedure that was previously performed by this group is followed. Experiments were performed in triplicate (Giray, 2019).

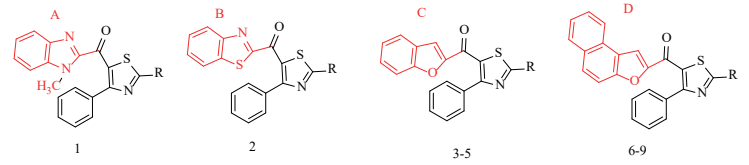
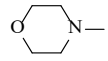
RESULTS AND DISCUSSION

Chemistry

Compounds were synthesized in a 55%-85% yield. Carbonyl peaks were observed around 1600 cm^{-1} and C-H stretchings were observed between 2800-3100 cm^{-1} in IR spectra. ^{1}H NMR and ^{13}C NMR spectral data were consistent with expectations. Compounds are consist of aromatic and aliphatic hydrogens. In the compound containing the 1-methylbenzimidazole structure, the hydrogens attached to the nitrogen attached to the methyl have a peak at 3.96 ppm in singlet form. Structures of the synthesized compounds are given below (Table 1).

Methylene hydrogens bound to the dialkylamine nitrogen have a peak around 3 ppm in ^{1}H NMR. The aromatic hydrogen peak in the five-membered regions of the benzofurane and naphthofurane rings is located between the peaks in the multiplet region, so the specific singlet is not separately observed. In ^{13}C NMR, the peak of the carbonyl carbon is observed between 173-175 ppm. The ^{13}C NMR peak of the methylene carbons adjacent to the dialkylamine nitrogen is observed at around 50 ppm. The methyl carbon attached to the 1st position of benzimidazole peaks was observed at 32.23 ppm. A carbon peak at the 2nd position of the thiazole was observed in the range of 164-171 ppm. Synthesized compounds are compatible with HRMS calculated data.

Table 1. Structures and some properties of the synthesized compounds

 1 2 3-5 6-9		Melting point °C	Yield	HRMS data (found)
1		230.3	72%	416.1786
2		176.1	57%	420.1199
3		166.8	65.5%	375.1167
4		152.8	67.7%	389.1318
5		199.3	69.8%	391.0508
6		181.7	71.3%	399.1158
7		186.3	64.6%	425.1321
8		169.1	83.9%	439.1477
9		186.7	77.5%	453.1649

Antibacterial activity

Antibacterial activity was tested by microdilution method against *E.coli*, *S.aureus,* and *Salmonella* species. Compounds did not show activity until 100 µM. Thus, compounds were evaluated as inactive. Antibacterial compounds generally have ionizable groups. In our tested compounds, amine groups are not good protonable groups as they are bound to aromatic thiazole ring. Besides, heterocyclic nitrogens are not protonable at medium pH's 4-9. These facts can be associated with the absence of the activity. For the other side of the molecules, in a former study, we have synthesized novel pyridine derivatives (Figure 2). Among them, 2 and 4- pyridyl derivatives showed low-moderate activity compared to chloramphenicol (Sahin, 2020). Following that, in this study, we have synthesized bicyclic heterocycles; however, this did not contribute to the activity. This can be the result of the blockade of the ring nitrogen hindrance by bicyclic structure, which would possibly interact with the probable target. Besides, in another study, Thomas et al. synthesized and tested similar structures. Similar to our study, they did not find activity on *S.aureus* and *E.coli*. They have discovered a close activity to penicillin for *B.subtilis* (Thomas, 2008a; Thomas 2008b).

In literature, antibacterial compounds are discussed for potential targets. There is numerous targets that change cell wall synthesis, protein synthesis, or some essential enzyme activities. Among these enzymes, compounds target the ATP binding site. ATP binding site ligands have amide, azomethine-NH, or protonable cyclic/acyclic moieties. In a detailed study performed on benzothiazoles mainly for *Mycobacterium tuberculosis*, potential antibacterial targets were evaluated with computational studies.

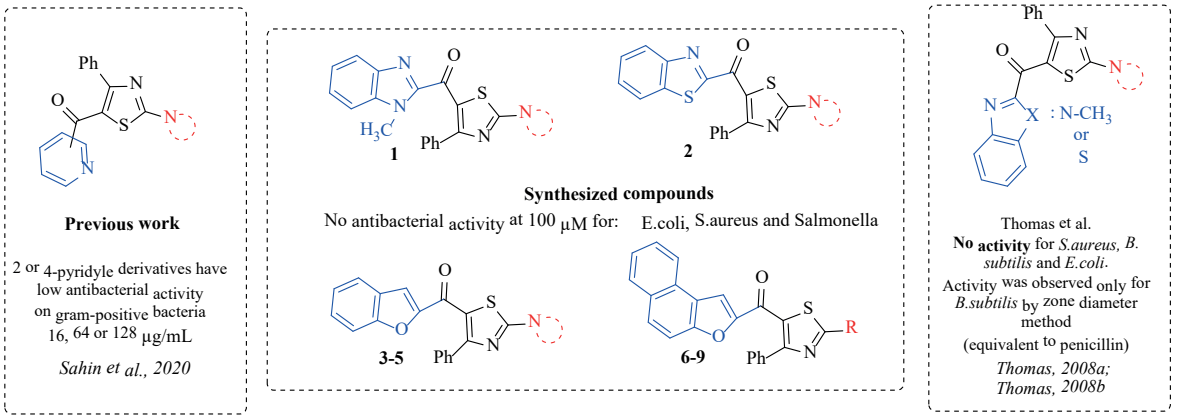


Figure 2. Summary of antibacterial activity

In this study, almost all of the active compounds bear carboxylic acid and/or amide moieties; thus, these or similar functional groups are essential for antibacterial activity (Gjorgjieva, 2018). Our synthesized structures do not have this kind of hydrophilic sites, which can also be the reason for the absence of antibacterial activity.

CONCLUSION

Consequently, nine novel thiazole derivatives were successfully synthesized and characterized. Although the similar compounds by this group were previously reported with different activities, this chemical group does not provide achievement in antibacterial drug development. Sidechains should be replaced and enriched with functional ionizable groups in the future.

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CONFLICT OF INTEREST

Authors declare there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Compounds were designed by S.D., and L.Y. Compounds were synthesized by Z.S., B.I.T., and E.A. Then structures were determined by L.Y., B.B., and Z.S. Antibacterial activity by microduliton method were made by A.C. Finally, manuscript preparation were realized by Z.S., E.A. and B.I.T.

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REVIEW ARTICLES

İlaç Endüstrisinde Proses Validasyonu ve Tasarımla Kalite (QbD) Yaklaşımı

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Process Validation in Pharmaceutical Industry and Quality by Design (QbD) Approach

SUMMARY

Process validation, which is defined as documented evidence that the process, operated within established parameters, can perform effectively and reproducibly to produce a medicinal product meeting its predetermined specifications and quality attributes. In the last decade, continuous process verification has been introduced, which is based on a continuous monitoring of manufacturing performance. This approach is based on the knowledge from product and process development studies and/or previous manufacturing experiences. Continuous process verification may be applicable to both a traditional and enhanced approach to pharmaceutical development. Process validation incorporates a lifecycle approach linking product and process development, validation of the commercial manufacturing process and maintenance of the process in a state of control during routine commercial production. Many pharmaceutical companies are adopting the principles of Quality by Design (QbD) for pharmaceutical development and manufacturing, which enables enhanced process understanding, and a more systematic and scientific approach to pharmaceutical development, so that better controls can be implemented. QbD is considered in examining validation within a product lifecycle framework. In this review, after reviewing the process validation approaches that are described in the current national and international guidelines, the focus will be on QbD and its significance in process validation.

Key Words: *Drug production, Process validation ,Quality by Design (QbD) ,PAT ,Continuous process verification*

İlaç Endüstrisinde Proses Validasyonu ve Tasarımla Kalite (QbD) Yaklaşımı

ÖZ

Proses validasyonu, belirlenmiş parametreler dahilinde yürütülen prosesin, önceden belirlenmiş spesifikasyonları ve kalite özelliklerini karşılayan bir tıbbi ürünü üretmek için etkin ve tekrarlanabilir bir şekilde performans gösterebildiğinin belgelenmiş kanıtıdır. Son on yılda üretim performansının sürekli izlenmesine dayanan kesintisiz proses doğrulaması ortaya atılmıştır. Bu yaklaşım, ürün ve proses geliştirme çalışmalarından ve/veya önceki üretim deneyimlerinden elde edilen bilgilere dayanmaktadır. Kesintisiz proses doğrulaması, farmasötik geliştirmeye yönelik hem geleneksel hem de daha gelişmiş bir yaklaşıma uygulanabilir. Proses validasyonu, ürün ve proses geliştirmesi, ticari üretim prosesinin validasyonu ve rutin ticari üretim sırasında prosesin kontrol altında tutulmasını birbirine bağlayan bir yaşam döngüsü yaklaşımını içermektedir. Çoğu ilaç firması, daha iyi bir kontrolün uygulanabilmesi için, ileri proses anlayışı ve daha sistematik ve bilimsel bir yaklaşıma dayalı sağlayan tasarımla kalite (QbD) ilkelerini benimsemektedir. QbD yaklaşımında validasyon, bir ürünün yaşam döngüsü çerçevesinde incelenmektedir. Bu derlemede, güncel ulusal ve uluslararası kılavuzlarda açıklanan proses validasyonu yaklaşımları gözden geçirildikten sonra, QbD ve proses validasyonundaki önemi üzerinde durulacaktır.

Anahtar kelimeler: *İlaç üretimi, Proses Validasyonu, Tasarımla Kalite (QbD), PAT, kesintisiz proses verifikasyonu*

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1. GİRİŞ

Ulusal ve uluslararası yayımlanan ilgili kılavuzlarda müstahzar, beşeri tıbbi ürün, tıbbi ürün, farmasötik ürün veya bitmiş ürün gibi farklı şekillerde ifade edilen "ilaç", hastalığı tedavi etmek ve/veya önlemek, bir teşhis yapmak veya bir fizyolojik fonksiyonu düzeltmek, düzenlemek veya değiştirmek amacıyla, insana uygulanan doğal ve/veya sentetik kaynaklı etkin madde veya maddeler kombinasyonu olarak tanımlanmaktadır (Beşeri Tıbbi Ürünler Ruhsatlandırma Yönetmeliği, 2005; Beşeri Tıbbi Ürünler İmalathaneleri Yönetmeliği, 2017). İlaç etkin maddesi ise, bir beşeri tıbbi ürünün üretiminde kullanılması planlanan, üretiminde kullanıldığında fizyolojik fonksiyonları düzeltmek, iyileştirmek, değiştirmek veya tıbbi teşhis amacıyla farmakolojik, immünolojik veya metabolik etki göstermek üzere ürünün etkin bileşeni olan madde ya da maddeler karışımı olarak tanımlanmaktadır (Beşeri Tıbbi Ürünler İmalathaneleri Yönetmeliği, 2017; EMA Glossary-Medicinal Product 2021).

İlacın nihai kullanıcı olan hastaya, istenen kalite, etkililik ve güvenlilikle ulaşması uluslararası ve ulusal kılavuzlar/yönetmeliklerle sağlanmaktadır. İlaçların güvenliliği ve etkililiği klinik araştırmalarla belirlenir. Klinik araştırmalar, bir veya birden fazla araştırma ürününün klinik, farmakolojik veya diğer farmakodinamik etkilerini ortaya çıkarmak ya da doğrulamak; advers olay veya reaksiyonlarını tanımlamak; emilim, dağılım, metabolizma ve atılımını tespit etmek; güvenliliğini ve etkililiğini araştırmak amacıyla insanlar üzerinde yürütülen çalışmalar olarak tanımlanmaktadır (İlaç ve Biyolojik Ürünlerin Klinik Araştırmaları Hakkında Yönetmelik, 2013). Klinik araştırmalar da ulusal ve uluslararası ilgili mevzuat doğrultusunda gerçekleştirilmektedir (İlaç ve Biyolojik Ürünlerin Klinik Araştırmaları Hakkında Yönetmelik, 2013; İyi Klinik Uygulamaları Kılavuzu, 2015). Bir etkin madde ve ilacın geliştirilmesi, üretilmesi ve raf ömrü sonuna kadar belirlenen özelliklere sahip olmasını için kalitesinin garanti edilmesi gerekmektedir.

Farmasötik kalite, bir ürünün, sistemin veya prosesin önceden tanımlanmış olan özelliklerine ait gereklilikleri karşılama derecesi olarak tanımlanmaktadır (*ICH Q10*, 2008). Bir ilaç üreticisini kalite açısından yönlendirmek ve kontrol etmek için oluşturulan farmasötik kalite sistemi, bir ürünün geliştirilmesinden teknoloji transferine, ticari üretimden üretimin durdurulmasına/sonlandırılmasına kadar olan süreci, diğer bir deyişle yaşam döngüsünü kapsamaktadır. Bu amaçla uluslararası ve ulusal yönetmelikler/kılavuzlarla tanımlanmış Farmasötik Kalite Sistemi uygulanmakta ve kalite bu gerekliliklere uyumla sağlanmaktadır (ICH Q10, 2008; Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu, 2018). Kalite güvence sistemi, ilacın beyan edilen amacına uygun olarak kullanımında gerekli olan kaliteyi temin etmek amacıyla düzenlenmiş uygulamalar bütünüdür. Ulusal mevzuat, üreticilerin etkin bir farmasötik kalite güvence sistemi kurmalarını ve yerine getirmelerini gerektirmektedir ve İyi İmalat Uygulamaları (İİU, Good Manufacturing Practice-GMP) Kılavuzunda Uluslararası Uyum Konferansı (ICH) terminolojisi ile tutarlı olmak adına Farmasötik Kalite Sistemi terimi kullanılmıştır. Dolayısıyla Kalite Güvence Sistemi ve Farmasötik Kalite Sistemi eşdeğer terimlerdir.

İİU, beşeri tıbbi ürün ve etkin maddelerin kullanım amacına uygun, ruhsatına esas bilgilerin veya ürün spesifikasyonunun gerekli gördüğü şekilde, kalite standartlarına uyumlu olarak sürekli imal edilmesini ve kontrolünü sağlayan kalite güvence sisteminin parçasıdır (Beşeri Tıbbi Ürünler İmalathaneleri Yönetmeliği, 2017).

Farmasötik Kalite Sistemi, bir ürünün tek başına veya toplu olarak kalitesini etkileyen tüm unsurları kapsayan, geniş çerçeveli bir kavramdır ve tıbbi ürünlerin amaçlanan kullanımları için gerekli kalitede olmasını sağlamak amacı ile yapılan organize düzenlemelerin bütünü anlamına gelmektedir(Commission Directive (EU) 2017/1572, 2017; Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu, 2018). Dolayısıyla kalite yönetimi, İİU'yu kapsamaktadır.

İİU prensipleri doğrultusunda, üretim yeri izin sahibinin tıbbi ürünlerin amaçlanan kullanımlarına uygun; ruhsatlandırma gerekliliklerinin sağlandığı; güvenlilik, kalite ve etkililik açısından hastaları riske atmayacak şekilde üretim gerçekleştirmesi hedeflenmektedir. İİU gereklilikleri ülkemizde yasal otorite olarak Türkiye İlaç ve Tıbbi Cihaz Kurumu (TİTCK) tarafından yürütülmektedir. TİTCK, Türkiye Cumhuriyeti Sağlık Bakanlığına bağlı olarak görev yapan ve Bakanlık politika ve hedeflerine uygun olarak ilaçlar, ilaç üretiminde kullanılan etkin ve yardımcı maddeler, ulusal ve uluslararası kontrole tabi maddeler, tıbbî cihazlar, vücut dışı tıbbî tanı cihazları, geleneksel bitkisel tıbbî ürünler, kozmetik ürünler, homeopatik tıbbî ürünler, insan vücuduna doğrudan temas eden biyosidal ürünler ve özel amaçlı diyet gıdalar hakkında düzenleme yapmakla görevli özel bütçeli, kamu tüzel kişiliğini haiz bir kamu kurumudur. Kasım 2011 tarihli 663 sayılı Kanun Hükmünde Kararname ile Sağlık Bakanlığının yapılanmasına ve sağlık sistemine ilişkin olarak yapılan köklü değişiklikler sonucunda Sağlık Bakanlığına bağlı İlaç ve Eczacılık Genel Müdürlüğü yerine yine Sağlık Bakanlığına bağlı olarak kurulmuştur (Sağlık Bakanlığı ve Bağlı Kuruluşlarının Teşkilat ve Görevleri Hakkında Kanun Hükmünde Kararname, 2011). TİTCK insan odaklı, bilimselliği esas alan değer üreten, uluslararası alanda öncü referans bir kurum olma hedefiyle ilerlemektedir.

İİU'ya dair düzenlemeler Amerika Birleşik Devletleri'nde Gıda ve İlaç Dairesi (FDA- Food and Drug Administration) tarafından, Avrupa'da ise Avrupa İlaç Ajansı (EMA-European Medicines Agency) tarafından yürütülmektedir. FDA, insan ve veteriner ilaçlarının, biyolojik ürünlerin ve tıbbi cihazların etkililik ve güvenliliğini sağlayarak halk sağlığını korumaktan sorumlu kuruluştur (FDA Mission, 2021). Benzer şekilde, EMA, ilaçları bilimsel standartlara göre değerlendirerek paydaşlara ilaçlar ile ilgili bağımsız ve bilimsel bilgiler sağlayarak halk ve hayvan sağlığını koruma misyonuyla Avrupa Birliği (AB) genelinde ve küresel olarak çalışmalar yürütmektedir. İlaçlar için üretim sahalarının İİU denetimlerinin koordinasyonu ile İİU faaliyetlerinin AB düzeyinde uyumlaştırılmasında önemli bir rol oynamaktadır (History of EMA, 2021).

Düzenleyici otoritelerin gerekliliklerinin uyumlaştırılması, otoriteler ve bölgeler arası farklı uygulamaların önüne geçilmesi amacıyla 1990 yılında, Avrupa, Japonya ve ABD düzenleyici otoritelerinin ve bu ülkelerdeki ilaç üreticileri birliklerinin bir araya gelmesiyle Uluslararası Uyum Konferansı (ICH: The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) kurulmuştur. 2015 yılında yapılan organizasyonel değişiklikler kapsamından ICH olarak yeniden yapılandırılmıştır. 2017 Kasım ayında Konseye gözlemci statüsünde katılan TİTCK, üyelik kriterlerinin tamamını yerine getirerek 27 Mayıs 2020 tarihinde düzenleyici üye otorite olarak kabul edilmiştir.

Tıbbi ürünler alanında uyumlaştırılmış İyi İmalat Uygulamaları standartlarının ve denetim otoritelerinin kalite sistemlerinin uluslararası gelişimini, uygulanmasını ve sürdürülmesini sağlamak amacıyla 1995 yılında Farmasötik Denetim İşbirliği Planı (PIC/S-Pharmaceutical Inspection Co-operation Scheme,) organizasyonu kurulmuştur (History of PIC/S, 2021). TİTCK da PIC/S üyeliğine 1 Ocak 2018 tarihi itibarıyla kabul edilmiştir. Bu doğrultuda, TİTCK, İİU gerekliliklerini, Dünya Sağlık Örgütü (WHO-World Health Organization), ICH, EMA, PIC/S gibi kuruluşlarca ve/veya ilgili diğer otorite ve organizasyonlar tarafından belirlenmiş kurallar çerçevesinde yürütmektedir.

İİU gerekliliği olarak bir ilacın önceden belirlenmiş spesifikasyonlar ve kalite özelliklerini karşılayacak şekilde tesis edilmiş parametreler dahilinde çalıştırılan bir prosesle tekrarlanabilir şekilde üretildiğinin proses validasyonu çalışmaları ile kanıtlanması gerekmektedir (Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu, 2018). Spesifikasyonlar, üretim boyunca kullanılan ya da elde edilen ürünlerin ya da materyallerin uymak zorunda oldu-

ğu gereklilikleri ayrıntılı olarak açıklamakta ve kalite değerlendirmesine esas oluşturmaktadır. Üretim prosesinde kullanılan sistemler ve ekipmanlar da onaylı proses yöntemleri ve ürün spesifikasyonları temel alınarak etkin ve tekrarlanabilir biçimde çalıştıkları kalifikasyon çalışmaları ile doğrulanmaktadır. Üreticilerin, ürünün ve prosesin yaşam döngüsü boyunca kalifikasyon ve validasyon statülerinin devamlılığını ve sürdürülebilirliğini sağlamaları bir İİU gerekliliğidir.

Proses validasyonu çalışmaları, FDA, EMA ve TİTCK otoritelerince belirlendiği üzere, geleneksel yaklaşım, kesintisiz proses doğrulaması ve hibrit olmak üzere üç farklı yöntem ile gerçekleştirilebilmektedir (Guideline on process validation for finished products, 2016; Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu, 2018). Kesintisiz proses doğrulaması, proses performansının kesintisiz olarak izlendiği ve değerlendirildiği bir alternatif proses validasyonu yaklaşımıdır (Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu, 2018). Yaşam döngüsü boyunca devam eden proses doğrulaması yaklaşımı ise her üç yaklaşıma da uygulanabilmektedir. Bu yaklaşım FDA kılavuzunda da proses validasyonunun 3. aşaması olarak ele alınmaktadır (Guidance for Industry Process Validation: General Principles and Practices 2011; Pazhayattil et al., 2018). Bu aşamada ürün kalitesinin sağlanması için sürekli veri toplanmaktadır Toplanan proses ve materyal verileri ile kontrol durumunun sürdürüldüğünü garanti etmek için ise istatistiksel kontrol araçları kullanılmaktadır.

Geleneksel proses validasyonunda, primer ambalajlama dahil tüm üretim aşamalarından geçen bitmiş ürün üzerinde yapılan testler ile ürünün kalitesi test edilmektedir. Bu durumda ürünün özellikleri ve kalitesi arasındaki ilişkiler tam olarak anlaşılması mümkün olmamaktadır. Bu yaklaşım, ürün kalitesi ile ilgili beklentileri tam olarak karşılamak yerine serilerin her birindeki farklılıkların saptanabilmesine katkı sağlamaktadır. Sonuç olarak, ürün kalitesinin daha çok test yapılarak artmayacağının görülmesi üzerine, tasarımla kalite 2008 yılında yayımlanan ICH Q8 kılavuzu ile resmi olarak gündeme gelmiştir. Otomotiv, petrokimya gibi diğer sanayi dallarında hâlihazırda kullanılmakta olan bu kavram böylece ilaç sektörüne de girmiştir. ICH Q8(R2) Kılavuzunda tasarımla kalite, "önceden tanımlanmış hedeflerle başlayıp, ürün ile prosesin sağlam bilim ve kalite risk yönetimine dayalı olarak anlaşılmasını vurgulayan sistematik bir ilaç geliştirme yaklaşımı" olarak tanımlanmış ve "kalite üründe test edilmez, kalite ürün içinde inşa edilmelidir" ifadesi kullanılmıştır (ICH Q8(R2) 2009; Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu, 2018). ICH Q8(R2) kılavuzunda ürün geliştirme ve üretim için "ampirik" ve "sistematik" olmak üzere iki farklı yaklaşımından bahsedilmektedir (ICH Q8(R2), 2009). Ampirik ürün geliştirme, geleneksel kabul edilen ürünlerin üretildikten sonra test edilmesine dayanan yaklaşım olarak belirtilmektedir. Tasarımla kalite yaklaşımı olarak da adlandırılan sistematik yaklaşımla ürün geliştirmede ise kapsamlı ürün ve üretim prosesi tasarımı kullanılmaktadır. Bu sistem ürünün yaşam döngüsü boyunca yenilenerek sürekli iyileştirilmesine olanak sağlamayı hedeflemektedir (ICH Q8(R2) 2009; Guidance for Industry Process Validation: General Principles and Practices 2011). Bu yaklaşımla, geliştirilen ve geliştirme sırasında belirlenen kontrol stratejisinin yüksek derece bir ürün kalite güvencesi sağladığının bilimsel olarak ortaya koyan kesintisiz proses doğrulaması kullanılmaktadır.

Bu yaklaşım kapsamında ilacın kalitesinde en büyük rolü oynayan üretim süreci ve bu süreçte göz önünde bulundurulan kalite özellikleri detaylı olarak göz önünde bulundurulmaktadır.

2. PROSES VALİDASYONU

İlaç endüstrisinde ürün kalitesi hayati öneme sahiptir. Bitmiş ürünün önceden belirlenmiş kalite özelliklerini karşıladığından emin olmak için üretim ile ilgili kritik parametrelerin belirlenmesi ve proses sırasında takip edilmesi son derece önemlidir (Singh, 2020). "Proses validasyonu" terimi henüz kullanımda

değilken üretilen farmasötik ürünler, kalitesi kontrol edilmeden doğrudan hastaya ulaştırılmış ve bu durum yan etkiler veya etkisizlik gözlenmesine sebep olmuştur. Karşılaşılan sorunlar, güvenlilik ve etkililik felaketlerini önlemek için kalite kontrol testlerinin, İİU'nun, klinik çalışmaların ve diğer kontrollerin oluşturulmasını sağlamıştır. Ancak ilaç üretim prosesleri zamanla daha karmaşık hale geldikçe sadece bitmiş ürünü test etmenin yeterli olmadığı ve ürünleri üretmek için kullanılan proseslere daha fazla önem verilmesi gerektiği fikri ortaya çıkmıştır. Validasyon kavramı ilk olarak 1979'da ABD'de iki FDA yetkilisi tarafından ilaçların kalitesini iyileştirmek için büyük hacimli parenteral ürünlerin sterilitesindeki bazı sorunlara doğrudan çözüm olarak önerilmiştir (Agalloco, 1995). İlk validasyon faaliyetleri, bu ürünlerin yapımında yer alan proseslere odaklanmış olup zamanla çevre kontrolü, besi yeri dolumu, ekipman sanitizasyonu/sterilizasyonu ve saf su/enjeksiyonluk su üretimi dahil olmak üzere ilgili proseslere de hızla yayılmıştır. 1982 yılında, 12 yaşında bir kız çocuğunun ailesi tarafından verilen Tylenol (asetaminofen) kapsülü almasının ardından birkaç saat içinde ölmesi ve aynı dönemde aynı aileden 3 kişi ve başkaları da dahil 6 kişinin aynı şekilde ölümünün gerçekleşmesi sonrası ürünün 31 milyon şişesi piyasadan geri çekilmiştir (Immel, 2001). İncelemeler sonucunda bir kişinin kapsülleri açtığı ve içine siyanür ekleyerek tekrar kapattığı ortaya çıkmış ve sonuç olarak 31 milyon şişe Tylenol imha edilmiştir. Bu olaydan sonra FDA tarafından önce ambalaj malzemeleri düzenlemesi üzerine ardından da değişik süreçler için bir seri düzenleyici kılavuz yayımlanmıştır. Bu kılavuzlara örnek olarak 1987 yılında yayımlanan proses validasyonu kılavuzu verilebilir (Guidance for Industry Process Validation: General Principles and Practices, 2011). 1987 yılında FDA'nın yayımlamış olduğu bu kılavuzda temel yaklaşım, prosesin üretim esnasında düzgün işlediğinden emin olmak için prosesi test etmek ve çalışmaya devam ettiğinden emin olmak için de üretim prosesinin periyodik olarak yeniden test edilmesi yönündedir (Guidance for Industry Process Validation: General Principles and Practices 2011; Immel, 2001) Ancak biyolojik/biyoteknolojik ürünlerin üretim proseslerinin, belirli bir aralık içinde test edilerek doğruluğundan emin olunamayacak kadar karmaşık yapıda olması nedeniyle bu kılavuzun geliştirilmesi gerekliliği ortaya çıkmıştır. Ayrıca, proses düzgün çalışmadığında derhal sinyal vermesini sağlayacak sistemlerin de geliştirilmesi gerektiği anlaşılmıştır. EMA ise 1996 yılında yayımladığı "Bitmiş Dozaj Şekillerinin İmalatı Hakkında Kılavuz"da ilk defa olarak proses validasyonu ifadesine yer vermiştir (Note For Guidance On Manufacture of the Finished Dosage Forms, 1996). Ardından 2000'lerin ortasında, endüstrinin yüksek kalitede ürün üretecek prosesler geliştirmesi için temel kavramları sağlamak adına ICH Q8 Farmasötik Geliştirme Kılavuzu hazırlanmıştır (ICH Q8(R2), 2009). 2009 yılında minör değişiklikler ile son halini alan, çok önemli bir proses validasyonu konseptini başlatan ve tasarımla kalite, tasarım alanı, kontrol stratejisi, gerçek zamanlı serbest bırakma testleri gibi kavramların tanımlandığı ICH Q8(R2) kılavuzu yayımlanmıştır (ICH Q8(R2), 2009).

Proses validasyonu, "Önceden belirlenmiş spesifikasyonları ve kalite özelliklerini karşılayan tıbbi ürünler üretmek için oluşturulmuş parametreler dâhilinde çalıştırılan prosesin etkin ve tekrar üretilebilir biçimde işlediğinin belgelenmiş kanıtıdır" şeklinde tanımlanmaktadır. Üretim prosesinin, bir ürünün geleneksel bir yaklaşımla veya gelişmiş bir yaklaşımla geliştirilip geliştirilmediğinden bağımsız olarak, valide edilmesi gerekmektedir (Guideline on process validation for finished products, 2016). Validasyon, piyasaya sürülecek ürünün üretimi için kullanılan tüm üretim aşamalarını ve tüm üretim sahalarını kapsamalıdır. Proses validasyonu, kontrol stratejisinin proses tasarımı ve ürünün kalitesi için yeterli olduğunu doğrulamalıdır. Bir ürünün farklı dozları, farklı seri ve ambalaj boyutları için yapılacak validasyon çalışmalarında bloklama (bracketing) yaklaşımı uygulanması mümkündür. Bloklama yaklaşımı, proses validasyonları esnasında yalnızca, doz, seri büyüklüğü ve/veya ambalaj büyüklüğü gibi önceden saptan-

mış ve gerekçelendirilmiş belirli tasarım faktörlerinin uç değerlerindeki serilerin test edildiği bilimsel ve risk bazlı bir validasyon yaklaşımıdır. Bu yaklaşım, herhangi bir ara seviyenin validasyonunun, uç değerlerin validasyonuyla temsil edildiğini varsaymaktadır. Farklı dozların valide edileceği durumlarda, dozlar özdeş veya bileşimleri birbirine çok yakınsa, bloklama uygulanabilmektedir. Örneğin, farklı baskı ağırlıklarındaki tablet gamına, farklı büyüklüklerdeki kapsül bloklarına veya aynı kap-kapak sistemindeki farklı dolumlara uygulanabilmektedir(Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu, 2018).

Güncellenmiş kılavuzlarda, geleneksel proses validasyonuna alternatif olarak üretim prosesi performansının sürekli olarak gözlemlenmesi ve değerlendirilmesi olarak tanımlanan kesintisiz proses doğrulaması yaklaşımı getirilmiştir (Guideline on process validation for finished products, 2016; Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu, 2018). FDA ve EMA otoriteleri tarafından yayımlanan kılavuzlarda proses validasyonu yaklaşımı temelde benzer olmakla birlikte yapısal olarak bazı farklılıklar göstermektedir (Tablo 1). FDA'nın ilgili kılavuzlarında, proses validasyonu üç temel aşamada ele alınırken, EMA kılavuzunda, ürün yaşam döngüsü üç temel aşamada açıklanmakta olup, proses validasyonu bu ürün yaşam döngüsünün ikinci aşaması olarak değerlendirmektedir. EMA kılavuzunda, FDA'dan farklı olarak, proses validasyonunun geleneksel, kesintisiz ve hibrit olmak üzere üç farklı yaklaşımla uygulanabileceği belirtilmektedir. Ülkemiz yasal ilaç otoritesi olan TİTCK da EMA yaklaşımına benzer olarak geleneksel, kesintisiz ve hibrit olmak üzere üç farklı proses validasyonu yaklaşımına yer vermektedir (Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu, 2018).

Tablo 1. FDA, EMA ve TİTCK kılavuzlarında belirtilen Proses Validasyonu Yaklaşımları

FDA	**EMA**	**TİTCK**
Proses Validasyonu Aşama 1: *Proses Tasarımı*	Ürün yaşam döngüsünün 1. Aşaması *Proses Tasarımı*	Ürün yaşam döngüsünün 1. Aşaması *Proses Tasarımı*
Proses Validasyonu Aşama 2: *Proses Kalifikasyonu*	Ürün yaşam döngüsünün 2. Aşaması *Proses Validasyonu* 1. Geleneksel Yaklaşım 2. Kesintisiz Proses Doğrulaması 3. Hibrit Yaklaşım	Ürün yaşam döngüsünün 2. Aşaması *Proses Validasyonu* 1. Geleneksel Yaklaşım 2. Kesintisiz Proses Doğrulaması 3. Hibrit Yaklaşım
Proses Validasyonu Aşama 3: *Devam Eden Proses Doğrulaması*	Ürün yaşam döngüsünün 3. Aşaması *Devam Eden Proses Doğrulaması*	Ürün yaşam döngüsünün 3. Aşaması *Devam Eden Proses Doğrulaması*

2.1 FDA Proses Validasyonu Yaklaşımı

FDA proses validasyonunu, bir prosesin sürekli olarak kaliteli ürün sunabileceğine dair bilimsel kanıt oluşturan, proses tasarım aşamasından ticari üretime kadar verilerin toplanması ve değerlendirilmesi olarak tanımlamaktadır (Guidance for Industry Process Validation: General Principles and Practices, 2011). Proses validasyonu, ürün ve prosesin yaşam döngüsü boyunca gerçekleşen faaliyetleri içermektedir. Proses validasyonu faaliyetleri proses tasarımı, proses kalifikasyonu ve devam eden prosesin doğrulaması olmak üzere üç aşamada açıklanmaktadır.

2.1.1 Proses Tasarımı (Aşama 1)

Proses tasarımı, planlanan ana üretim ve kontrol kayıtlarına yansıtılacak olan ticari üretim prosesini tanımlama faaliyetidir. Ticari üretim prosesi, geliştirme ve ölçek büyütme (scale up) faaliyetleriyle kazanılan bilgilere dayalı olarak bu aşamada tanımlanır.

Bu aşamanın amacı, kalite özelliklerini karşılayan bir ürünü sürekli olarak üretebilecek, rutin ticari üretime uygun bir proses tasarlamaktır.

2.1.2 Proses Kalifikasyonu (PQ) (Aşama 2)

Bu aşamada, prosesin yeniden üretilebilir ticari üretim yeteneğine sahip olup olmadığını belirlemek için proses tasarımı değerlendirilir. Bu aşamanın, tesisin tasarımı ile ekipman/yardımcı tesislerin kalifikasyonu ve proses performans kalifikasyonu (PPQ) olmak üzere iki unsuru vardır. Ticari ürünlerin serbest bırakılmasından önce Aşama 2'nin başarıyla tamamlanması gerekmektedir ve üretilen ürünler bu aşamada uygun bulunursa dağıtım için serbest bırakılabilir.

Tesisin Tasarımı ve Ekipman /Yardımcı Tesislerin Kalifikasyonu

Bir üretim tesisinin uygun tasarımı, İİU Kılavuzu Tesisler ve Ekipman Bölümü düzenlemeleri ve kılavuzun ilgili bölümleri kapsamında, PPQ'dan önce ve ticari ölçekte ürün üretmeden gerçekleştirilmesi beklenmektedir. Burada kalifikasyon terimi, yardımcı tesislerin ve ekipmanların amaçlanan kullanımları için uygun olduğunu ve düzgün bir şekilde çalıştığını göstermek için yapılan faaliyetleri ifade etmektedir.

Proses Performans Kalifikasyonu (PPQ)

PPQ, proses kalifikasyonunun ikinci unsuru olup, ticari seriler üretmek için fiili tesisi, yardımcı tesisleri, her birinin kalifikasyonları tamamlanmış durumda olan ekipmanları ve eğitimli personeli, ticari üretim prosesi, kontrol prosedürleri ve bileşenleri birleştirmektedir. Başarılı bir PPQ, proses tasarımını onaylayacak ve ticari üretim prosesinin beklendiği gibi işlediğini gösterecektir. Bu aşamadaki başarı, ürünün yaşam döngüsünde önemli kilometre taşlarından biri olarak kabul edilmektedir. Bir üretici, ilaç ürününün ticari dağıtımına başlamadan önce PPQ'yu başarıyla tamamlamalıdır. Ticari dağıtıma başlama kararı, ticari ölçekli serilerden gelen verilerle desteklenmelidir. Laboratuvar ve pilot çalışmalardan elde edilen veriler ise ticari üretim sürecinin beklendiği gibi çalıştığına dair sadece ek güvence sağlayabilir.

PPQ, bilime, üreticinin genel ürün ve proses anlayışı ile kanıtlanabilir kontrollere dayanmalıdır. Tüm ilgili çalışmalardan elde edilen kümülatif veriler (örneğin, tasarlanmış deneyler, laboratuvar, pilot ve ticari seriler) PPQ'daki üretim koşullarını oluşturmak için kullanılmalıdır.

2.1.3 Devam Eden Proses Doğrulaması (Aşama 3)

Validasyonun 3. aşamasının amacı, ticari üretim sırasında prosesin kontrol altında (valide durumda) kaldığına dair sürekli güvence sağlanmasıdır. Bu amaca ulaşmak için, proseste meydana gelebilecek planlanmamış sapmaları tespit etmek için sistemlerin tasarlanmış olması esastır. Proses performansının değerlendirilmesi, sorunları tanımlamak ve prosesin kontrol altında kalması için sorunları öngörmek, önlemek ve düzeltmek için eylemde bulunulması gerekip gerekmediğini belirlemektedir. Ürün kalitesiyle ilgili ürün ve proses verilerini toplamak ve analiz etmek için devam eden bir program oluşturulmalıdır. Toplanan veriler, ilgili proses eğilimlerini ve gelen malzemelerin veya bileşenlerin kalitesini, in-proses malzemeleri ve bitmiş ürünleri içermelidir. Veriler istatistiksel yönelimli olmalı ve eğitimli personel tarafından incelenmelidir. FDA tarafından veri toplama planını, proses stabilitesini ve proses yeteneğini (process capability, Cp) ölçme ve değerlendirmede kullanılan istatistiksel yöntemler ile prosedürleri geliştirmede istatistikçinin veya yeterli eğitimi olan bir kişinin görev alması tavsiye edilmektedir.

İyi proses tasarımı ve geliştirme için, önemli değişkenlik (varyasyon) kaynaklarını önceden tahmin etme ve uygun tespit, kontrol ve/veya hafifletme stratejilerinin yanı sıra uygun uyarı ve aksiyon limitleri oluşturulmalıdır. Bununla birlikte, bir prosesin daha önce tespit edilmemiş veya prosesin daha önce maruz kalmadığı varyasyon kaynaklarıyla karşılaşması muhtemeldir. Varyasyonu tespit etmek, karakterize etmek ve temel nedeni belirlemek için istatistiksel ve/veya daha nitel olan birçok araç ve teknik, kullanılabilir. Seri içi olduğu kadar seriler arası varyasyonun da incelenmesi kapsamlı bir sürekli proses doğrulama programının parçasıdır (Beşeri Tıbbi Ürünler İma-

lathaneleri İyi İmalat Uygulamaları Kılavuzu, 2018). Proseste varyasyon, kalite şikayetlerinin, spesifikasyon dışı bulguların, proses sapma raporlarının, proses verim varyasyonlarının, seri kayıtlarının, gelen hammadde kayıtlarının ve advers olay raporlarının zamanında değerlendirilmesiyle de tespit edilebilmektedir. Bu aşamada toplanan veriler, prosesin veya ürünün çalışma koşulları, proses kontrolleri, bileşen veya inproses malzeme özellikleri gibi bazı yönlerini değiştirerek prosesi iyileştirme ve/veya optimize etme yolları önerebilir. Önerilen değişikliğin ürün kalitesini nasıl etkileyebileceğine bağlı olarak, ek proses tasarımı ve proses kalifikasyon faaliyetleri gerekli görülebilir.

2.2 EMA Proses Validasyonu Yaklaşımı

EMA Proses Validasyonu yaklaşımına göre ürünün yaşam döngüsü üç aşamada ele alınmaktadır (Guideline on process validation for finished products, 2016). İlk aşama, ICH Q8(R2) Kılavuzunda tanımlanan proses tasarımı, ikinci aşama EMA Proses Validasyonu kılavuzunda detayları ile de belirtilen proses validasyonu aşaması, üçüncü aşama ise GMP Kılavuzu Ek 15'de ele alınan devam eden proses doğrulaması aşamasıdır. EMA Proses Validasyonu Kılavuzu; ICH Q8(R2)'de açıklandığı gibi etkin bir kalite sistemi altında ve risk yönetimi araçlarıyla birlikte gelişmiş proses anlayışını uygularken üreticilerin ICH Q8, Q9 ve Q10 da açıklanan yeni yaklaşımlardan nasıl yararlanabileceklerini açıklamak amacıyla yayımlanmıştır.

Proses validasyonu, ürün geliştirme yaklaşımından bağımsız olarak geleneksel bir yaklaşımla (geleneksel proses validasyonu) gerçekleştirilebilir. Diğer bir alternatif olarak, ürün geliştirme aşamasında ileri bir yaklaşım uygulanmışsa veya geçmiş veriler ve üretim deneyimi yoluyla önemli miktarda ürün ve proses bilgisi ve anlayışı kazanılmışsa, kesintisiz proses doğrulaması uygulanabilir. Geleneksel proses validasyonu ve kesintisiz proses doğrulamasının bir kombinasyonu (hibrid yaklaşım) da kullanılabilmektedir. Aşağıda bu yaklaşımlardan daha detaylı olarak bahsedilecektir.

2.2.1 Geleneksel Proses Validasyonu

Geleneksel proses validasyonu normalde farmasötik geliştirme ve/veya proses geliştirme tamamlandığında, ölçek büyütme aşamasından sonra ve bitmiş ürünün piyasaya sürülmesinden önce gerçekleştirilmektedir (Guideline on process validation for finished products, 2016). Proses validasyonu yaşam döngüsünün bir parçası olarak, ölçek büyütmeden önce pilot ölçekteki seriler üzerinde proses validasyonu çalışmaları yürütülebilmektedir. Pilot seri boyutu, üretim ölçeğindeki serinin en az %10›u olmalıdır, diğer bir deyişle ölçek büyütme için çarpma faktörü 10'u geçmeyecek şekilde olmalıdır. Katı oral dozaj şekilleri için pilot seri boyutu genellikle maksimum üretim boyutunun %10'u veya 100.000 birim (hangisi daha büyükse) olmalıdır. Diğer dozaj şekilleri için pilot seri boyutunun, hasta yönüyle dozaj şeklinin başarısız olma riski dikkate alınarak gerekçelendirilmesi gerekmektedir. Pilot ölçekli seriler üzerinde tam validasyon çalışmaları yürütmenin genel olarak yararlı olmadığı düşünülmektedir. Üretim ölçeğinde (ticari ölçekte) her ürün için tam validasyon çalışması tamamlanmalıdır ve bu çalışmalarda bloklama yaklaşımı kabul edilebilmektedir.

Seri sayısı, prosesin değişkenliğine, prosesisin/ürünün karmaşıklığına, geliştirme sırasında kazanılan proses bilgisine, teknoloji transferi sırasında ticari ölçekte destekleyici verilere ve üreticinin genel deneyimine dayanmalıdır. Aksi gerekçelendirilmedikçe, en az üç ticari ölçekli seriye ait veriler sunulmalıdır. Ancak bir gerekçe sunulduğu durumlarda, bir veya iki ticari ölçekli seriye ilişkin verilerin, bunların pilot ölçekli serilerine ait validasyon verileri ile desteklenmesi geleneksel proses validasyonu yaklaşımında yeterli görülebilmektedir. Bununla birlikte, ilerleyen bölümlerde detayları verildiği üzere, TİTCK geleneksel proses validasyonu yaklaşımında en az üç ticari ölçekli seri gerekliliği yer almakta olup gerekçe sunularak pilot ölçekli seri(ler) ve üçten daha az ticari ölçekli seri(ler) ile proses validasyonunun tamamlanması hususu yer almamaktadır.

Geleneksel yaklaşımın kullanıldığı üretim prosesleri için mümkün olan durumlarda ürünün ruhsatlandırılmasından önce, ileriye dönük (prospektif) bir validasyon programından geçmesi gerekir. Bununla birlikte; hasta lehine güçlü bir yarar-risk oranının bulunduğu istisnai durumlarda, rutin üretimin başlamasından önce bir validasyon programının tamamlanmaması kabul edilebilir ve eşzamanlı validasyon kullanılabilir. Ayrıca, tekrar üretim çalışmalarından alınan verilerin mevcut olmadığı, sınırlı sayıda etkin madde serisinin üretildiği, etkin madde serilerinin düzensiz aralıklarla üretildiği veya etkin madde serilerinin değiştirilmiş bir valide işlem tarafından üretildiği zamanlarda da yapılabilmektedir. Eş zamanlı validasyon, validasyon protokolünün validasyon serilerinin ticarileştirilmesiyle eşzamanlı olarak icra edildiği istisnai koşullarda yapılan, anlamlı hasta yararının bulunması temelinde gerekçelendirilen validasyon olarak tanımlanmaktadır.

Geriye dönük validasyon (retrospektif) ise artık kabul edilebilir bir yaklaşım olarak görülmemektedir (Guideline on process validation for finished products, 2016; Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu, 2018).

2.2.2 Kesintisiz Proses Doğrulaması

Kesintisiz proses doğrulaması, üretim süreci performansının sürekli olarak izlendiği ve değerlendirildiği geleneksel proses validasyonuna alternatif bir yaklaşımdır (ICH Q8(R2), 2009). Tasarımla kalite yaklaşımıyla geliştirilen ve geliştirme sırasında oluşturulan kontrol stratejisinin yüksek derecede bir ürün kalite güvencesi sağladığının bilimsel olarak ortaya konulduğu durumlarda, geleneksel proses validasyonuna bir alternatif olarak kesintisiz proses doğrulaması kullanılabilmektedir (Eudralex Volume 4 EU Guidelines for Good Manufacturing Practice for Medicinal Products for Human and Veterinary Use Annex 15: Qualification and Validation, 2015; Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu, 2018). Kesintisiz proses doğrulaması, önceden tanımlanmış parametreler kapsamında işleyen bir proses-le tutarlı bir şekilde tüm kritik kalite özelliklerini ve kontrol stratejisi gereksinimlerini karşılayan bir ürün üretildiğini doğrulamak ve göstermek için bilime ve riske dayalı gerçek zamanlı bir yaklaşımdır (Guideline on process validation for finished products, 2016). Kesintisiz proses doğrulamasını uygulamak için, üreticilerin kapsamlı in-line, on-line ve at-line kontroller gerçekleştirmesi ve her seride proses performansını ve ürün kalitesini izlemesi gerekmektedir. Başlangıç materyallerin veya bileşenlerin, proses içi materyallerin ve bitmiş ürünlerin kalite özelliklerine ilişkin veriler toplanmalıdır. Bu veriler, kalite özelliklerinin, parametrelerin ve bitiş noktalarının doğrulanmasını ve kritik kalite özelliklerini ve kritik proses parametreleri (critical process parameters-CPP) eğilimlerinin değerlendirilmesini de içermelidir. Yakın infrared (NIR) spektroskopisi ve Çok Değişkenli İstatistiksel Proses Kontrolü (MSPC) gibi proses analitik teknolojisi (PAT) uygulamaları, kesintisiz proses doğrulamasının uygulanmasını kolaylaştıran araçlardır.

Kesintisiz proses doğrulamasının desteklenmesi için, daha önce benzer ürün veya proseslerin geliştirilmesi ve üretilmesi sırasında elde edilen bilgiler, ürünün geliştirme çalışmaları ve ticari üretim deneyimlerinden kazanılan proses anlayış oranı, ürün ve/veya üretim prosesinin karmaşıklığı, proses otomasyonu ve analitik teknolojilerin kullanılma seviyesi gibi hususlarda yeterli seviyede proses bilgisi ve anlayışı gereklidir (Guideline on process validation for finished products, 2016). Kesintisiz proses doğrulaması stratejisinin uygunluğu ve fizibilitesine ilişkin bilgiler, izlenecek proses parametreleri ve malzeme özelliklerinin yanı sıra kullanılacak analitik yöntemler de dahil olmak üzere validasyon dosyasına dahil edilmeli ve en azından laboratuvar veya pilot ölçekli serilerden elde edilen verilerle desteklenmelidir. Üretim ölçeğinde kesintisiz proses doğrulaması sırasında üretilen gerçek veriler, inceleme için tesiste mevcut olmalıdır. Başvuru sahibi, ürünün piyasaya sürülmesinden önce prosesin kontrol altında sayıldığı aşamayı ve tamamlanan validasyon çalışmasını ve bu kararın hangi esasa göre verileceğini tanımlamalıdır. İlgili dokümanlar,

prosesin karmaşıklığına, beklenen değişkenliğine ve üretim tesisinin mevcut üretim deneyimine dayalı olarak kullanılacak parti sayısı için bir gerekçe içermelidir. Kesintisiz proses doğrulaması, kesintisiz prosesleri valide etmek için en uygun yöntem olarak kabul edilmektedir.

Kesintisiz proses doğrulaması, ürünün yaşam döngüsü içinde herhangi bir zamanda uygulanabilir. İlk ticari üretim için, ticarileştirilmiş ürünleri proses değişikliklerinin bir parçası olarak yeniden valide etmek veya sürekli iyileştirmeyi desteklemek için kullanılabilir.

2.2.3 Hibrit Yaklaşım

Üretim prosesindeki farklı aşamalar için geleneksel proses validasyonu veya kesintisiz proses doğrulaması yaklaşımlarını birlikte kullanmanın gerekli olduğu durumlarda tek bir üretim prosesi için hibrit yaklaşım benimsenebilmektedir (Guideline on process validation for finished products, 2016; Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu, 2018). Bu durumda üretim prosesindeki hangi adım için hangi validasyon yaklaşımının kullanıldığının açıkça belirtilmesi gerekmektedir (Guideline on process validation for finished products, 2016). Validasyonun gereklilikleri seri boyutu ve seri sayısı açısından kesintisiz proses doğrulamasının ne ölçüde kullanıldığına göre belirlenmektedir. Kesintisiz proses doğrulamasının kullanılmadığı proses basamaklarında geleneksel proses validasyonu yaklaşımının uygulanması, aksi halde gerekçelendirilmesi gerekmektedir.

Bu yaklaşım aynı zamanda, değişiklikler sonrası herhangi bir validasyon aktivitesi için veya ürün başlangıçta geleneksel yaklaşım kullanılarak valide edilmiş olsa da devam eden proses doğrulaması boyunca da kullanılabilmektedir.

2.2.4 Yaşam Döngüsü Boyunca Devam Eden Proses Doğrulaması

Proses validasyonunun üçüncü aşaması olarak tanımlanan ve devam eden proses doğrulaması olarak da tanımlanan yaşam döngüsü boyunca devam eden proses doğrulaması, geleneksel, kesintisiz ve hibrit yaklaşım olan her üç proses validasyonu yaklaşımına da uygulanabilmektedir (Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu, 2018). Yaşam Döngüsü Boyunca Devam Eden Proses Doğrulaması yaklaşımında validasyon hiçbir zaman tamamlanmayıp, sürekli devam etmektedir. Üreticiler, ürünün yaşam döngüsü boyunca bir kontrol durumunun sürdürüldüğünü garanti etmek için ürün kalitesini, ilgili proses verilerinin yönelimlerinin değerlendirilmesiyle birlikte izlemektedir. Ürün yaşam döngüsünün herhangi bir noktasında, proses kavrayışının ve proses performansının mevcut seviyesini hesaba katarak gereksinimler uygulanmaktadır. Belirli bir prosesin değişkenliğine ve kapasitesine ilişkin tüm sonuçların desteklenmesi ve bir kontrol durumunun garanti edilmesi amacıyla istatistiksel araçlar kullanılmaktadır.

İstatistiksel araçlardan biri olan İstatistiksel Proses Kontrolü (Statistical Process Control-SPC); bir prosesin kararlı, öngörülebilir ve istatistiksel kontrolde olup olmadığını belirlemek için kullanılabilmektedir ("Technical Report No. 60 Process Validation: A Lifecycle Approach," 2013). Bu kontrol, proses yeteneğinin (C_p) istatistiksel olarak hesaplanması ile uygulanmaktadır (Eşitlik 1).

$$C_p = \frac{(USL - LSL)}{6s} \qquad \textit{(Eşitlik 1)}$$

USL, üst spesifikasyon limiti (Upper Specification Limit),

LSL, alt spesifikasyon limitini (Lower Specification Limit),

s: standart sapma

Validasyonun ve devam eden proses doğrulamasının amacı, hem seri içi hem de seriler arası varyasyonları belirlemektir. C_p, prosesin spesifikasyonları tutarlı bir şekilde karşılayıp karşılamadığını belirlemek için kullanılmaktadır. Örneğin; 20 dakikada bir alınan tablet ağırlığı verileri için veya her saat başı yapılan pH kontrolleri için C_p değeri kullanılmaktadır. Eğer sadece belirli bir seriye ait ve o seriyi temsil

eden veriler varsa, C_p değerleri hesaplanır ancak örneğin bir yıl içerisinde üretilen tüm serilere ait veriler mevcut ise proses performans (P_p) değerleri hesaplanmaktadır. Pp hesabı için de yine Eşitlik 1 de verilen denklem kullanılmaktadır, ancak bu defa standart sapma içerisinde hesaplanacak olan veriler belirli bir uzun döneme ait (örn: 1 yıl) veriler olacaktır. Hesaplama sonucu ortaya çıkan veri, proses performansını ortaya koymaktadır. Dolayısıyla üretilen her yeni serinin verileri ile P_p değerleri değişkenlik gösterecektir. P_p değerleri prosesin geleceği ile ilgili bilgi vermeyip, üretilen seriler ile elde edilen verilerin istatistiksel olarak hesaplanması sonucu prosesin kontrol altında olup olmadığı hakkında bir bilgi sağlamaktadır.

2.3 TİTCK Proses Validasyonu Yaklaşımı

TİTCK, İİU kapsamında EMA ile benzer proses validasyonu yaklaşımını benimsemektedir. EMA'da olduğu gibi ve yukarıda detayları verildiği şekilde İİU Kılavuzunda da geleneksel proses validasyonu, kesintisiz proses doğrulaması ve hibrit yaklaşım olmak üzere 3 başlık altında tanımlanmaktadır (Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu, 2018). Bu kılavuzda, proses validasyonu ile ilgili gerekliliklerin, yukarıda bölüm 2.2'de bahsedilen EMA Proses Validasyonu Kılavuzu gereklilikleri ile birlikte değerlendirilmesi gerektiği belirtilmektedir.

Geleneksel proses validasyonu yaklaşımında, tekrar üretilebilirliği doğrulamak amacıyla, bitmiş ürünün birkaç serisinin rutin üretim şartları altında üretildiği ve üretilen en az üç ardışık serinin, prosesin istikrarlı biçimde kaliteli ürün ortaya koyma yeteneğinde bulunduğu gösterilmektedir (Eudralex Volume 4 EU Guidelines for Good Manufacturing Practice for Medicinal Products for Human and Veterinary Use Annex 15: Qualification and Validation, 2015). Üretimdeki standart yöntemlerin kullanılıp kullanılmadığını ve tesiste hâlihazırda benzer ürünlerin veya proseslerin kullanılıp kullanılmadığını hesaba katarak alternatif bir seri sayısının da geçerliliği ispatlanabilmektedir. Üç seri ile yapılacak bir başlangıç validasyon uygulamasının, devam eden proses doğrulaması uygulamasının parçası olan ardışık serilerden elde edilen verilerle desteklenmesi de gerekebilir. Her bir üretici, prosesin istikrarlı biçimde kaliteli ürün ortaya koyma yeteneğinde bulunduğunu yüksek bir güvence seviyesiyle göstermeye yeterli olacak seri sayısını belirlemeli ve bunu gerekçelendirmelidir.

ICH Q8(R2) kılavuzu ile hayatımıza giren yeni yaklaşım, proses validasyonunun tek seferlik bir olay olarak görülmemesi gerektiğini ve proses validasyonunun ticari ürünün valide edilmesi ve ticari üretim devam ettiği sürece prosesin kontrol altında olması için ürün ve proses geliştirme arasında bir bağ kuran yaşam döngüsü yaklaşımını içermesi gerektiğini vurgulamaktadır (ICH Q8(R2) 2009; Guideline on process validation for finished products, 2016). Bu doğrultuda, üretim proseslerinin geliştirilmesinde kesintisiz proses doğrulaması yaklaşımı, geleneksel proses validasyonu yaklaşımının yerine veya onunla birlikte kullanılabilmektedir (Guideline on process validation for finished products, 2016; Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu, 2018).

İİU Kılavuzunda ana hatları verilen prensipler ve gereklilikler tüm farmasötik dozaj şekillerinin üretimine uygulanabilir. Bunlar, yeni proseslerin ilk validasyonunu, modifiye edilen proseslerin sonraki validasyonunu, tesis transferlerini ve devam eden proses doğrulamasını kapsamaktadır. Etkili bir proses validasyonu ilaç kalitesinden emin olunmasına önemli bir katkı sağlamaktadır.

3. TASARIMLA KALİTE (QbD - QUALITY BY DESIGN)

İlaç endüstrisi güvenilir, düşük maliyetli, sağlam ve minimum safsızlığa sahip ürün sağlama sorumluluğunda olduğu için, üretim sürecinde meydana gelebilecek sorunların olabildiğince erken tespit edilmesi gerekmektedir (Manzon, 2020). Bu nedenle etkin madde ve ürünlerin üretiminde yukarıdaki bölümlerde anlatıldığı şekilde geleneksel kalite anlayışı tasarımla kalite (QbD) anlayışına dönüşmüştür. Zaman içinde QbD ile ürün özellikleri ve proses parametre-

lerinin kritiklik durumuna göre bir risk değerlendirme çalışması yapılması gerektiği anlaşılmış ve "Kalite Risk Yönetimi" ve bunu takiben "Farmasötik Kalite Sistemi" ile ilgili kılavuzlar yayımlanmıştır (ICH Q9, 2005; ICH Q10, 2008).

QbD, önceden tanımlanmış hedeflerle başlayıp, ürün ile prosesin bilimsel temellere ve kalite risk yönetimine dayalı olarak anlaşılmasını vurgulayan, sistematik bir ilaç geliştirme yaklaşımı olarak tanımlanmaktadır (ICH Q8(R2) 2009). QbD, önceden belirlenmiş kalitenin sağlanması için, ürün tasarlama ve ürün geliştirme boyunca kullanılacak ilgili üretim proses aşamalarını kapsar. Bilgi yönetimi ile kalite risk yönetimi ise QbD'nin uygulanmasında temel unsurlardır. QbD, kılavuzlarda tanımlanan proses validasyonunun tamamlayıcı unsurlarındandır.

3.1. QbD Elementleri

Genel olarak, ilaç ürünü geliştirme ve üretimi, ön formülasyon, prototip formülasyonu, pilot ya da temel ölçekli üretim gibi birkaç aşamada gerçekleşmektedir. QbD' nin uygulanması için, formülasyon ve üretim proses değişkenlerinin ürün kalitesini nasıl etkilediğinin anlaşılması gerekmektedir. Kritik formülasyon özelliklerini mevcut üretim prosesi seçenekleri ile birlikte değerlendirmek, üretim sürecinin seçimini ele almak ve bileşenlerin uygunluğunu onaylamak yönüyle önemlidir. Proses geliştirme çalışmaları, prosesin iyileştirilmesi, proses validasyonu, sürekli proses doğrulaması ve proses kontrol gereklilikleri için temel kabul edilmektedir. Proses geliştirme çalışmalarından elde edilen bilgi, bitmiş ürün spesifikasyonlarını doğrulamak için de kullanılmaktadır. QbD, temelde aşağıda belirtilen elementlerden oluşmaktadır:

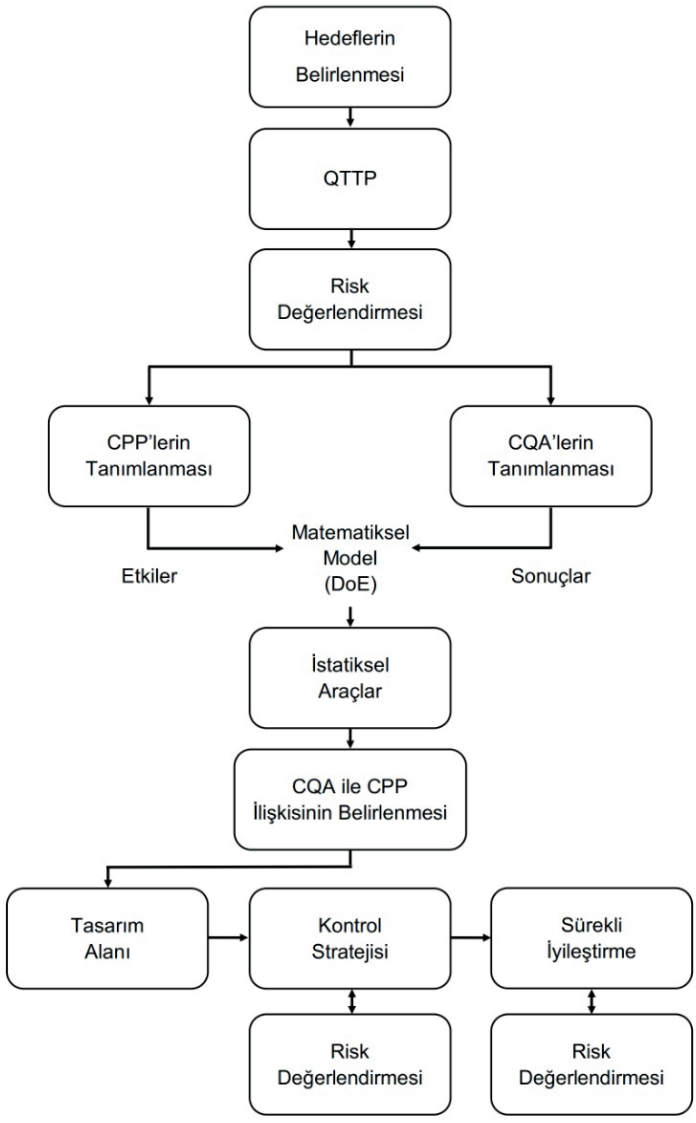
- Hedef Ürün Kalite Profili (Quality Target Product Profile- QTPP)
- Kritik Kalite Özellikleri (CQA)
- Kritik Materyal Özellikleri (Critical Material Attributes-CMA)
- Kritik Proses Parametreleri (CPP)
- Kontrol Stratejisi
- Tasarım Alanı
- Deney Tasarımı (DoE)
- Risk değerlendirmesi

QbD uygulanmasında uygulanan aşamalar ve QbD elementleri Şekil 1' de özetlenmiştir.

QbD yaklaşımında ilk adım, Kalite Hedef Ürün Profilini (QTPP-Quality Target Product Profil) belirlemektir (Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu, 2018; Dahmash et al., 2018; Pallagi et al., 2019; Manzon, 2020)). Sonraki aşamada, CQA ve CPP ile aralarındaki ilişki belirlenerek tasarım alanı oluşturulmaktadır. Belirlenen tasarım alanı içinde prosesin kontrol altında olduğundan emin olunması için risk değerlendirmesi çalışmaları ile uygun bir kontrol stratejisi oluşturulması gerekmektedir. Kontrol stratejisi oluşturulmuş bir proses ile üretilen ürünün yaşam döngüsü boyunca da risk değerlendirmesi çalışmaları ile sürekli iyileştirilmesi sağlanmaktadır.

Hedef Ürün Kalite Profili (QTPP):

Hedef ürün profilini ve bunun kalite kriterlerini tanımlayan QTPP, ilaç ürününün güvenliliği ve etkililiği hesap edilerek, ideal olarak istenen kaliteyi sağlamak için elde edilecek kalite özelliklerinin ileriye dönük özetidir (ICH Q8(R2), 2009). QTPP seçimi, klinik beklentiler, hasta ve endüstrinin ihtiyaçları, düzenleyici otoritenin bakış açısı gibi ilgili tarafların gereksinimlerine dayanmaktadır. QTPP, uygulama yolu, dozaj şekli, dağıtım sistemleri ve kap kapak sistemi gibi genel özellikleri içerebilmektedir. Bununla birlikte çözünme gibi farmakokinetik özellikler ile saflık ve stabilite gibi kalite kriterlerini de içerebilmektedir. Ürüne özel örnekler için transdermal sistem için yapışma kuvveti veya topikal bir sistem için viskozite söylenebilir. QTPP spesifikasyon değildir çünkü spesifikayon serinin serbest bırakılması için seride yapılmayan biyoeşdeğerlik veya stabilite gibi testleri içermektedir (Aksu, 2013).



Şekil 1. QbD aşamaları ve elementleri (Dahmash et al., 2018' den uyarlanmıştır)

Kritik Kalite Özellikleri (CQA):

İstenen ürün kalitesini güvence altına almak için onaylanmış limit, aralık veya dağılım dâhilinde olması gereken; fiziksel, kimyasal, biyolojik veya mikrobiyolojik özellik veya ayırt edici nitelik olarak tanımlanmaktadır (ICH Q8(R2) 2009; Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu, 2018; Pallagi, 2019; Manzon, 2020). Hedef ürün kalitesi üzerine kritik etkisi olduğu bilinen CQA'ların seçimi önemlidir ve bu parametrelerin seçiminde geçmişe dayanan bilgi ve deneyimleri temel alınması gerekmektedir. CQA'ya ilişkin kabul kriterlerinin, hem geleneksel proses validasyonu yaklaşımında hem de QbD yaklaşımında belirlenmesi gerekmektedir.

Kritik Materyal Özellikleri (CMA):

Ürünün CQA'sı üzerinden etkili olan etkin madde ve yardımcı maddelerin fiziksel, kimyasal ve biyolojik özellikleridir. Ürünün geliştirilmesi sırasında etkin maddenin polimorfik formu ve partikül büyüklüğü gibi çözünürlüğünü doğrudan etkileyebilecek fiziksel özelliklerinin göz önünde bulundurulması gerekmektedir. Diğer yandan etkin maddenin kimyasal stabili-

tesi de üretim prosesini şekillendirebilmektedir. Bununla birlikte hedeflenen ürün profiline ulaşabilmek için yardımcı madde ve bileşenlerin de etkin madde ile uyumlu olacak şekilde seçilmesi gerekmektedir. Dolayısıyla ürün performansından emin olunması için uygun kontrollere karar verilebilmesi adına ürünün geliştirilmesi aşaması boyunca kritik materyal özellikleri her bileşen için tanımlanmalıdır (Pazhayattil et al., 2018).

Kritik Proses Parametreleri (CPP):

CPP, değişkenliğin kritik kalite özelliği üzerinde etkisinin olması nedeniyle istenen kalitede ürünün üretildiğini güvence altına almak için, proseste izlenmesi veya kontrol edilmesi gereken proses parametreleridir (ICH Q8(R2) 2009; Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu, 2018). Üretim proseslerinin aynı olduğu durumlarda dahi CPP'ler ürünün çeşidine, materyallerin özelliklerine ve ürünün hedef profiline bağlı olarak farklılık gösterebilmektedir (Pallagi et al., 2019).

Kontrol Stratejisi:

Mevcut ürün ve prosesin anlaşılmasından elde edilen ve proses performansı ile ürün kalitesini güvence altına alan planlı kontroller dizisi olarak tanımlanan kontrol stratejisi, üretim boyunca ürünün gerektirdiği kalitenin sağlamlık ve tutarlılığını garanti etmek için gereklidir. CMA, CQA ve CPP için bilimsel temelli bir kontrol stratejisinin olması gerekmektedir (*ICH Q10*, 2008). Üretim prosesinin geliştirilmesi aşamasında, proses izleme verilerinin toplanarak prosesin geliştirilmesi için faydalı bilgiler sunarak, tanımlı olması gereken tüm kritik özelliklerin kontrolünü sağlayarak prosese bir doğrulama kapasitesi sağlamaktadır (ICH Q8(R2), 2009).

Kontrol stratejisi oluşturulurken, üretim süreci ile proses kontrollerinin tanımı, malzemelerin kontrolü, kritik adımların ve ara ürünlerin kontrolleri ve ambalajlama işlemlerinin de kontrol stratejisi çalışmasına dahil edilmesi beklenmektedir (*ICH Q11,* 2012).

Bir kontrol stratejisi, hammaddeler, başlangıç malzemeleri, ara ürünler gibi malzeme özelliklerini ve bunlar üzerindeki kontrolleri, tesis ve ekipman operasyon koşullarını, üretim tasarımındaki kontrol süreçlerini, in-proses testler ve proses parametreleri gibi proses kontrollerini, seri serbest bırakma testi gibi ilaç üzerindeki kontrolleri içermektedir, ancak bunlarla sınırlı değildir (*ICH Q10*, 2008; *ICH Q11, 2012*). Kontrol stratejisi, her bir ilacın seri serbest bırakma spesifikasyonlarının ilaç kalitesini sağlamak için uygun aralık içinde ve/veya sınırda olmasını sağlamalıdır. Böylece ürünün istikrarlı bir şekilde istenilen kalitede üretilmesini sağlamaktadır(*ICH Q11, 2012*).

Kontrol stratejisi dokümantasyonu, toplanan ve monitorize edilen üretim verilerinin tüm CQA'ların saptandığını ve kontrol altında olduğunu gösterdiğini garantilemek için farmasötik kalite sistemi ile de ilişkilendirilmelidir (Aksu, 2013).

Tasarım Alanı (Design Space):

Kalite güvencesini sağladığını gösteren girdi değişkenlerinin, örneğin materyal özelliklerinin ve proses parametrelerinin çok boyutlu kombinasyonu ve etkileşimi olarak tasarım alanı, CQA ile onları etkileyen CPP arasındaki çok değişkenli işlevsel ilişkileri tanımlar ve birim operasyonları ile olan bağlantılarını içine alır (Aksu, 2013,ICH Q8(R2), 2009).

Tasarım alanının dışına çıkılması bir değişiklik olarak görülmekte olup, düzenleyici otoritenin onayını gerektirmektedir. Bu doğrultuda ruhsat sonrası varyasyon başvurusunu gerektirmektedir (ICH Q8(R2), 2009). Tasarım alanında tüm girdiler ürünün kalitesini değiştirmeden değişebilmektedir. Tasarım alanını tanımlamak için mekanik modeller ile birlikte çeşitli matematiksel modeller de kullanılabildiği için formülasyon tasarımı ve proses ölçek büyütme işlemleri sırasında riskleri azaltması nedeniyle üstünlük sağlamaktadır. Bununla birlikte tasarım alanı düzenleyici otorite tarafından zorunlu görülmemektedir (Quality Implementation Working Group on Q8, Q9 and Q10 Questions & Answers (R4) 2010; Pazhayattil et al., 2018). Bir proses kontrol stratejisini doğrulamak

amacıyla, eğer kullanıldıysa tasarım alanının geçerliliğinin gösterilmesinin ve kullanılan bir matematiksel modelin geliştirilmesinin altında yatan proses bilgisinin mevcut olması gerekmektedir (Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu, 2018). CMA ve CPP için yapılan tüm DoE çalışmalarının sonuçlarının birleşimi tasarım alanını tanımlamaktadır. İstatistiksel araç olarak kullanılan deney tasarımı (DoE) ise risksiz karar almak için güvenilir bilgi sağlamaktadır (Manzon, 2020).

Deney Tasarımı (DoE):

Ürün geliştirme prosesi temelde, iki ana unsur olan malzeme ve üretim prosesinin anlaşılması ve bunlarla ilgili sahip olunan bilgiye dayanmaktadır ve bu nedenle ürün geliştirme aşaması boyunca CQA ile ilaç arasındaki ilişki incelenmektedir. Bu amaçla yararlanılan QbD elementlerinden biri olan DoE, kalite güvencesini sağladığı gösterilmiş olan proses parametreleri ve girdi değişkenlerinin (ör. materyel özellikleri) çok yönlü kombinasyonu ve etkileşimi olarak tanımlanmaktadır (ICH Q8(R2), 2009). Proses koşulları ile kontrol parametreleri arasındaki etkileşimi tam olarak anlayabilmek için gerekli parametreleri değiştirerek çok sayıda deney yapmak gerekmektedir. DoE, gerekli bilgileri elde etmek için yapılması gereken deneyleri mümkün olduğunca verimli ve kesin bir şekilde planlamak, tasarlamak ve analiz etmek için istatistiki düşüncenin uygulanmasını sağlayan sistematik bir yaklaşım olanağı sunmaktadır. Bu yaklaşım ile tek seferde tek bir değişkenin etkisini incelemek yerine matematiksel modeller yardımıyla, prosesi etkileyebilecek çok sayıda deneysel değişkenleri daha verimli olarak incelenebilmektedir (Pharmaceutical Quality by Design A Practical Approach, 2018; Zacché & Andersson, 2019).

Geleneksel proses validasyonu yaklaşımı ile yukarıda detayları verilen QbD elementlerinin karşılaştırılması Tablo 2'de verilmiştir.

Tablo 2. Proses validasyonunda geleneksel yaklaşım ile QbD yaklaşımı kullanılarak kesintisiz proses doğrulaması uygulamasının karşılaştırılması

Elementler	**Geleneksel Proses Validasyonu**	**QbD Yaklaşımı ile Kesintisiz Proses Doğrulaması**
Tasarım Alanı	Yok	Var
Kalite Hedef Ürün Profili (QTPP)	Var	Var
Kritik Kalite Özellikleri (CQA)	Var	Var
Kritik Proses Parametreleri (CPP)	Belirlenen parametreler daha genel	Belirlenen parametreler daha detaylı
Proses Anlayışı	Yüzeysel	Değişkenlerin ürüne ve prosese etkisi geniş çerçevede, daha detaylı bir bakış açısıyla değerlendirildiği için daha derin bir proses anlayışı
Kontrol Stratejisi	Testlere ve denetime dayalı bir kontrol stratejisi mevcut	Daha ileri (gerçek zamanlı serbest bırakma, çevrimiçi test etme gibi) ve riske dayalı uygulama
Proses Analitik Teknolojisi (PAT)	Yok	Var
Risk değerlendirilmesi	Kalite Yönetiminin bir parçası olması gerektiği için var, ancak daha az kullanılır	Daha sık ve etkin şekilde kullanılır

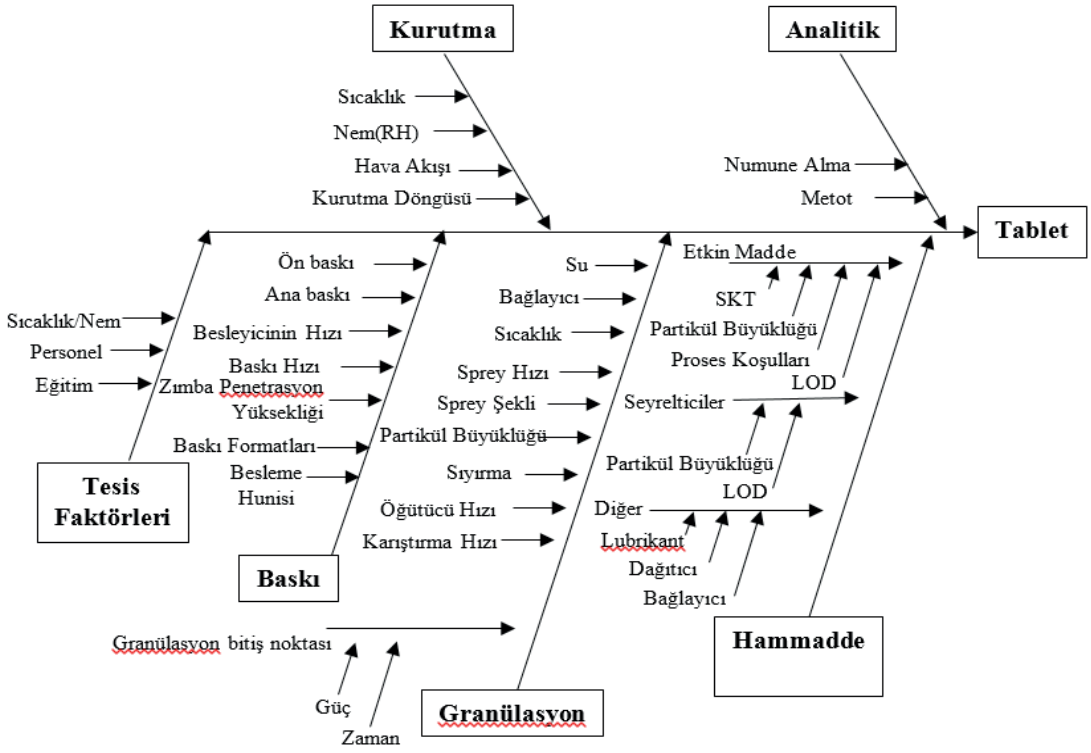
Risk Değerlendirmesi

Yaşam döngüsü boyunca kaliteye dair risklerin değerlendirilmesine, kontrolüne, iletişimine ve gözden geçirilmesine yönelik sistematik proses, Kalite Risk Yönetimi olarak tanımlanmaktadır. (ICH Q9, 2005; Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu, 2018)

Etkin bir kalite sisteminin değerli bir bileşeni olan kalite risk yönetimi, tehlikelerin tanımlanması ve bu tehlikelere maruz kalma ile ilgili risklerin analiz edilmesi ve değerlendirilmesidir (*ICH Q9*, 2005). Risk değerlendirilmesi, kaliteye yönelik potansiyel riskleri belirlemek, bilimsel olarak değerlendirmek ve kontrol etmek için proaktif bir yaklaşım sağlayan ve bununla birlikte, yaşam döngüsü boyunca ürün ve proses performansında sürekli iyileştirmeyi kolaylaştıran önemli araçlardan biridir (Patil & Pethe, 2013).

Kalite Risk Yönetimi, ürünü ve prosesi etkileyen kritik noktalara odaklanılmasını sağladığından, QbD yaklaşımının geliştirilmesi ve uygulanmasında temel elementlerden biridir (Patil & Pethe, 2013). Şekil 1 ve Tablo 3'de belirtildiği üzere, QbD yaklaşımının uygulandığı bir ürün geliştirme sürecinde, Kalite Risk Yönetimi sıklıkla ve etkin bir şekilde uygulanmaktadır. Kalite Risk Yönetiminin, kaliteye yönelik riskin değerlendirilmesinin bilimsel bilgiye dayanması ve nihayetinde hastanın korunmasına bağlanması ve Kalite Risk Yönetimi prosesi için harcanan enerji ve hazırlanan dokümantasyonun riskin düzeyi ile orantılı olması şeklinde iki temel prensibi bulunmaktadır (ICH Q9, 2005; Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu, 2018). Risk değerlendirmesi, iyi tanımlanmış bir problemin açıklanması veya bir risk sorusu sorulması ile başlamaktadır. Risk iyi tanımlandığında, uygun risk yönetimi aracı ve risk sorusunu ele almak için gerekli bilgi türleri daha kolay tanımlanmaktadır.(*ICH Q9*, 2005). Bu nedenlerle ilk aşama olan ve "Ne yanlış gidebilir?", "Yanlış gitme olasılığı nedir?" ve "Sonuçları, şiddeti ne olur?" sorularının sorulduğu riskin tanımlanması aşaması risk değerlendirmesinin temelini oluşturmaktadır. Risk değerlendirmesinin ikinci aşaması olan riskin analiz edilmesi, riskin oluşma olasılığı ile zararın ciddiyeti arasında bağlantı kurmanın niteliksel veya niceliksel süreci olarak tanımlanmaktadır. Bazı risk değerlendirme araçlarında tespit edilebilirlik, analiz aşamasının bir faktörü kabul edilmektedir. Son aşama olan değerlendirme aşamasında tespit edilen ve analiz edilen riskler risk kriterleri ile karşılaştırılır. Etkili bir risk değerlendirmesinde verilerin sağlamlığı, çıktıların kalitesini etkileyeceği için çok önemlidir. Kalite risk yönetiminde kullanılabilecek araçlara örnek olarak Başarısızlık Durumu Etkilerinin Analizi (FMEA), Hata Ağacı Analizi (FTA), Tehlike Analizi ve Kritik Kontrol Noktaları (HACCP), Tehlike İşletilebilirlik Analizi (HAZOP), Ön Tehlike Analizi (PHA), Risk Sıralaması ve Filtreleme ve Destekleyici İstatistiksel Gereçler ve Balık Kılçığı Analizi (veya Ishikawa Diyagramı) gösterilebilmektedir (Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu, 2018; Pazhayattil et al., 2018). Ishikawa diyagramı, potansiyel hatalara ait birçok olası nedeni tespit etmek, dokümante etmek ve muhtemel problemle ilişkili olan kök neden analizi için kullanılmaktadır. Bu diyagram aynı zamanda söz konusu etkiye yol açan olası faktörler arasındaki ilişkileri de tanımlamaya yarar (Aksu, 2013). Buna bir örnek olarak, Şekil 2'de tablet hazırlanması için risk değerlendirmesi çalışmasında kullanılan Ishikawa diyagramı verilmiştir.

Risk değerlendirmesi ürünün gelişim aşamasında farklı zamanlarda yeniden yapılmalı ve ürünün ticari yaşam döngüsü boyunca bir risk yönetimi uygulamasına dönüşmelidir. Bu uygulama üretimde verimliliğin artırılmasına olduğu kadar ürün kalite güvencesini sağlamak için kullanılan kontrol stratejisi elemanlarının sürekli geliştirilmesine olanak sağlar.



Şekil 2. Tablet hazırlanması için Ishikawa (balık kılçığı) diyagramı örneği (*ICH Q8(R2)* 2009)

3.2 Gerçek Zamanlı Serbest Bırakma Testleri (RTRT- Real Time Release Testing)

Etkin madde ve bitmiş ürünün belirlenen kalitede olup olmadığının anlaşılması için spesifikasyonları karşıladığının test edilmesi gerekmektedir (*Guideline on Real Time Release Testing (formerly Guideline on Parametric Release)*, 2012). Bu sebeple etkin madde ve bitmiş ürün üzerinde birçok test yapılması gerekmektedir. Ancak bu yaklaşıma bir alternatif olarak sistematik testler uygulanabilmektedir. Bu yaklaşım, ilk başlarda parametrik serbest bırakma adı altında ve ilgili kılavuz çerçevesinde yalnızca son kabında sterilize edilen ürünlerin sterilite testleri için uygulanmıştır. 2012 yılında ise EMA tarafından yayımlanan kılavuz ile bu yaklaşım yeniden şekillenmiştir (*Guideline on Real Time Release Testing (formerly Guideline on Parametric Release)*, 2012). ICH Q8, Q9 ve Q10 kılavuzları ile de tanımlanan bu yeni yaklaşım, sterilite dışındaki testlere de uygulanabilen parametrik serbest bırakmaya benzer bir serbest bırakma karar prosesi olup Gerçek Zamanlı Serbest Bırakma Testleri (RTRT) olarak adlandırılmaktadır. Dolayısıyla, belirli koşullar altında, yetkili otorite tarafından izin verilen durumlarda, ürün bilgisine ve proses kavrayışına dayanarak üretim prosesi boyunca elde edilen veriler, bitmiş ürün seri serbest bırakma testleri yerine kullanılabilmektedir (*Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu*, 2018). Ayrıca bu seri serbest bırakma şeklinin gerektirdiği faaliyetlerin tümünün farmasötik kalite sistemi ile bütünleştirilmesi gerekmektedir. RTRT, genellikle ölçülen materyal özellikleri ile proses kontrollerinin valide bir kombinasyonunu içeren proses verilerine dayanan in-proses ve/veya bitmiş ürün kalitesini değerlendirebilmek ve garanti edebilmek olarak tanımlanmaktadır (*ICH Q8(R2),* 2009).

RTRT uygulanması, üreticiye bitmiş ürün testinin/testlerinin azaltılabilmesi gibi üstünlükler sağlarken, düzenleyici otorite bakış açısından da olumlu olarak karşılanmaktadır (*Guideline on Real Time Release Testing (formerly Guideline on Parametric Rele-*

ase), 2012). Ancak RTRT, QbD'nin uygulanması için düzenleyici otorite tarafından zorunlu tutulmamaktadır (*Quality Implementation Working Group on Q8, Q9 and Q10 Questions & Answers (R4)* 2010). RTRT, in-proses izlemelerin ve kontrollerinin bir kombinasyonu olarak, izin verildiği takdirde, seri serbest bırakma kararı kapsamında bitmiş ürün testlerine alternatif teşkil edebilmektedir (*Beşeri Tıbbi Ürünler İmalathaneleri İyi İmalat Uygulamaları Kılavuzu*, 2018). Onaylı RTRT ile kontrol edilen bitmiş ürün özelliklerinin ilgili serilerin analiz sertifikalarında bulunması istenmektedir ve RTRT ile kontrol edilen parametreler için "RTRT ile kontrol edilmiştir" şeklinde dipnot düşülmesi gerekmektedir. Bu yaklaşımın uygulanması, belirli bitmiş ürün testi/testleri kapsamında olup, bitmiş ürün üzerinde yapılan testlerin tamamen kaldırılacağı anlamına gelmemektedir (*Quality Implementation Working Group on Q8, Q9 and Q10 Questions & Answers (R4)* 2010). Örneğin, seri serbest bırakma testlerinden spesifik olarak izin alınan bir test için RTRT uygulanabilirken diğer testler için rutin seri serbest bırakma testlerinin gerçekleştirilmesi gerekliliği devam ettiğinden, seri serbest bırakma testlerinden yalnızca RTRT uygulanmasına izin verilen test için bu yaklaşım uygulanabilmektedir. Örneğin, dağılma (disintegrasyon) testi, yüksek oranda çözünür etkin maddeler ile hızlı dağılan katı dozaj şekilleri için çözünme (dissolüsyon) testi yerine kullanılabilir. In-proses olarak gerçekleştirilen birim doz tekdüzeliği (örneğin NIR testiyle) gerçek zamanlı serbest bırakma testi olarak kullanılabilir (ICH Q8). Diğer bir örnek ise son kabında sterilize edilen steril ürünler için, seri serbest bırakma testlerinden sterilite testi için kullanılan ve RTRT nin de bir şekli olan parametrik serbest bırakmada ise bitmiş üründe yapılan ve örneklem olarak seçilen numunelerde gerçekleştirilen ve oldukça zaman alan sterilite testi yerine proses parametrelerinin izlemine (örneğin: terminal sterilizasyon için sıcaklık, basınç, süre parametrelerinin gerçek zamanlı izlemine) dair belgelerin gözden geçirilmesi ve değerlendirilmesi yeterli görülebilmektedir.

3.3 Proses Analitik Teknolojisi (PAT)

PAT, "Kalite üründe test edilmez, kalite ürün içinde inşa edilmelidir" olarak tanımlanan QbD'nin etkin şekilde uygulanmasına yardımcı olan önemli araçlardan biridir (Zhang & Mao, 2017). PAT'ın hedefi, üretim prosesinin anlaşılmasını ve kontrolünü geliştirmektir. Başka bir ifadeyle, etkin maddeye, in-proses materyallere ve proseslere ait kritik kalite ve performans özelliklerinin zamanında ölçülerek üretimin tasarlanması, analiz ve kontrol edilmesi için kurulan bir sistemdir (ICH Q8(R2), 2009).

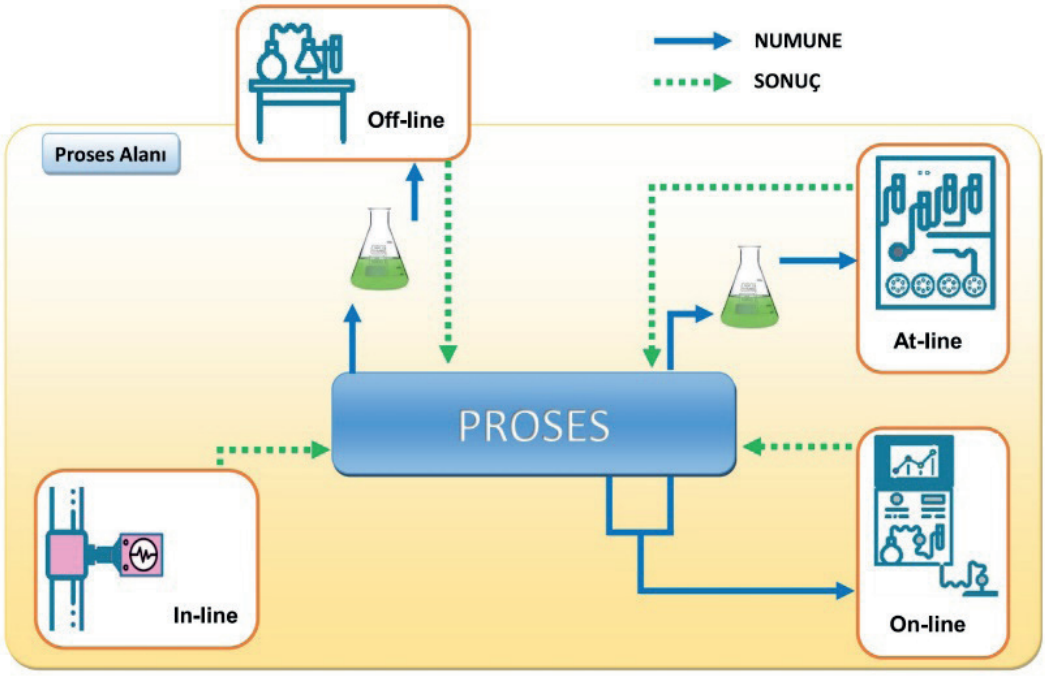
PAT teriminde yer alan "analitik" ifadesi, kimyasal, fiziksel, mikrobiyolojik ve matematiksel yöntemler ile risk analizinin entegre şekilde uygulanmasını içermektedir (*Pharmaceutical Quality by Design A Practical Approach*, 2018). PAT bir analiz cihazı değil, gerçek zamanlı bilgiye dayalı aksiyon alınmasını sağlayan bir sistemdir. Bu gerçek zamanlı bilgi, bir proses analizi cihazından, bir matematiksel modelden ya da laboratuvar testinden elde edilebilir ancak mutlaka "gerçek zamanlı" olmalıdır.

PAT, aynı zamanda, CQA, CMA veya CPP'nin kontrol edilmesini sağladığından kontrol stratejisinin önemli bir parçasıdır (Pramod et al., 2016). Ürün formülasyonunun ve üretim prosesinin ürün performansını nasıl etkilediği ile ilgili mekanik bilgi eksikliği, "Ürün Profili-Kritik Kalite Özellikleri-Risk Değerlendirmesi-Tasarım Alanı-Kontrol Stratejisi-Sürekli Gelişim" QbD zincirinin bir noktada kopmasına neden olmaktadır (Celik, 2018). Başarılı bir risk değerlendirmesi, formülasyon bileşenlerinin fizikokimyasal ve mekanik özellikleri hakkında derinlemesine bilgi sahibi olunmasını, her ünit operasyonun mekaniğinin anlaşılmasını, etkin madde, eksipiyanlar ve proses parametreleri arasındaki etkileşimin tanımlanmış olmasını gerektirir. Bu bağlamda, spesifikasyonların mekanik anlayışa bağlı olarak belirlenmesi ve prosesin kontrol edilebilmesi için gerçek zamanlı serbest bırakma ve prosesi sürekli geliştirme imkanı sunan PAT araçlarının kullanılması çok önemlidir (*PAT — A Framework for Innovative Pharmaceutical*

Development, Manufacturing, and Quality Assurance Guidance for Industry, 2004; *Pharmaceutical Quality by Design A Practical Approach*, 2018).

Yeni bir ürün veya proses tasarlamak, endüstriyel üretimi kontrol etmek, ürünün yaşam döngüsü boyunca proses anlayışını kazanmak, teknoloji transferi yapmak ve ürünün spesifikasyonlar dahilinde sağlam bir prosesle üretildiğinden emin olmak için farklı PAT araçları kullanılmaktadır (*Pharmaceutical Quality by Design A Practical Approach*, 2018). Bu araçlar, proses analizörleri (çok değişkenli), proses sensörleri (tek değişkenli), proses modelleme araçları, deney tasarımı (DoE) ve çok değişkenli veri analizleri (kemometri) olarak sıralanabilir.

Geliştirme aşamasında prosesin anlaşılması ve üretim aşamasında prosesin kontrol edilmesi için çok önemli olan gerçek zamanlı ölçümler, proses sensörleri ve proses analizörleri tarafından gerçekleştirilmektedir. Proses sensörleri; sıcaklık, basınç, iletkenlik, pH, hız, zaman ve sıklık gibi tek değişkenli verileri ölçüm cihazı ile okumaktadır. Proses analizörleri ise; analitik yazılımlar, veri yönetim sistemleri ve ölçüm ara yüzü içeren çok değişkenli sistemlerdir (Clegg & Biospin, 2020). Bazı proses analizörleri, işlem gören malzemenin kimyasal, fiziksel ve biyolojik özellikleri ile ilgili bilgi veren ve malzemeye zarar vermeyecek şekilde ölçümler yapabilmektedir (*PAT-A Framework for Innovative Pharmaceutical Development, Manufacturing, and Quality Assurance Guidance for Industry*, 2004). Bu ölçümler aşağıda detayları ve şematik gösterimi verildiği şekilde at line, in-line veya on-line olabilmektedir (*PAT-A Framework for Innovative Pharmaceutical Development, Manufacturing, and Quality Assurance Guidance for Industry*, 2004; *Pharmaceutical Quality by Design A Practical Approach*, 2018).



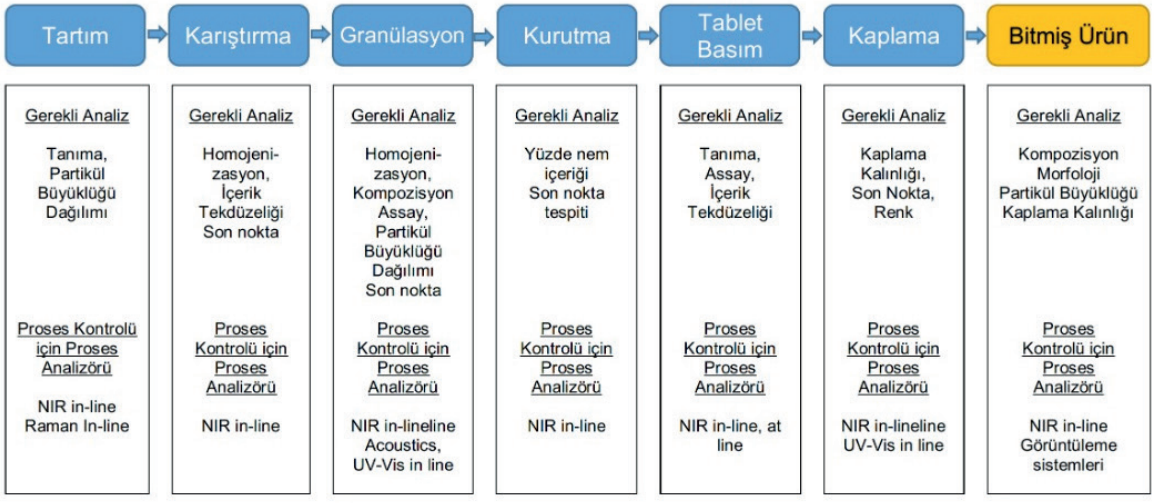
Şekil 3. At line, in-line, on-line ve off-line ölçümlerin şematik gösterimi (*Bu terimler dilimizde de aynı şekilde kullanılmakta olduğu için İngilizce olarak verilmiştir)*

At-line ölçümlerde, proses akışı sırasında numune alındıktan sonra proses akışına yakın bir yerde analiz edilmekte ve genelde dakikalar içinde sonuç alınabilmektedir.

On-line ölçümlerde, numune, proses akışından analize yönlendirilmekte ve proses akışına geri dönebilmektedir. Bu şekilde prosese müdahale olmadan ölçüm yapılarak saniyeler içinde sonuç alınabilmektedir.

In-line ölçümlerde, numune proses akışından uzaklaştırılmadan invazif veya invazif olmayan şekilde ölçüm yapılabilmektedir. Örneğin; bir proses ekipmanının penceresinden bir prob yardımıyla ölçüm alınması halinde prob, proses metaryeli ile doğrudan temas halinde olabilir. Bu durumda ölçüm sonuçlarının alınması saniyeleri bile bulmamaktadır.

Şekil 4'te katı dozaj şekli üretiminde PAT uygulamasına bir örnek verilmiştir. PAT uygulamasında spektroskopik ölçümler, kullanım kolaylığı, hızlı sonuç vermesi ve taşınabilir olması üstünlükleri ile önemli rol oynamaktadır (Monakhova et al., 2017). Near Infrared (NIR) araçları, hem sıvılara hem de toz ve katılara uygulanabilmesi nedeniyle üretim prosesinin kontrolü için en çok kullanılan bir spektroskopik yöntemdir. Bu teknolojiyle kritik proses ve ürün özelliklerinin gerçek zamanlı ölçümlerinde numune hazırlanmadan hızlı ölçüm ile anlık sonuç alınabilmektedir (*Pharmaceutical Quality by Design A Practical Approach*, 2018). Raman spektroskopi yöntemi de NIR gibi katı dozaj formu üretiminde kullanılan, numune hazırlamadan ve materyale zarar vermeden, fiziksel ve kimyasal özelikler ile ilgili hızlı sonuç veren PAT teknolojilerinden biri olarak kullanılmaktadır (Vanhoorne & Vervaet, 2020).



Şekil 4. Katı Dozaj Şekli Üretiminde Unit Operasyonlarında Kullanılan Proses Analizörlerine Örnekler (*Pharmaceutical Quality by Design A Practical Approach*, 2018' dan uyarlanmıştır)

3.4 Proses validasyonunda QbD uygulanmasının üstünlükleri

Yaklaşık son 10 yıldır ilaç üretiminde benimsenen farklı aşamalardan oluşan proses validasyon yaklaşımları, hedeflenen kalitede bir ilaç ürününün üretilmesinde önemli üstünlükler getirmiştir. QbD temelli ürün geliştirme, tasarım ve sürekli proses doğrulaması aşamaları detaylı bir planlama ve yatırım gerektirmekle birlikte, genel olarak düşünüldüğünde hasta sağlığını ilgilendiren paydaşlar (hasta, endüstri, resmi otorite) açısından da en yararlı yaklaşım olmuştur. Bu yaklaşımla, üretimde olası hataları en düşük seviyelere indirerek hastaya kaliteli ürün ulaştırılması sağlanırken diğer iki paydaşa da yarar sağladığı aşikardır (Beg et al., 2019).

İlaç endüstrisi açısından bakıldığında, tasarımla kalite yaklaşımı üst düzeyde ve derinlemesine bir proses kavrayışı ve ürün anlayışı sağladığı için serilerin reddedilmesi veya geri çekilmesi durumlarını ciddi ölçüde azaltmaktadır (Beg et al., 2019; Zacché & Andersson, 2019). QbD yaklaşımının önemli elementlerinden biri olan tasarım alanının uygulanması ile prosesin ölçek büyütme işlemleri sırasında daha etkili ve verimli kontrol sağlanmaktadır. Bununla birlikte, risk değerlendirmesinin daha sık ve etkin şekilde kullanımı ile riskleri azaltmak kolay hale gelmekte, bu durum verimi arttırmakla birlikte ürün geliştirme için harcanan zaman, para ve iş gücünden tasarruf edilmesini sağlamaktadır.

Diğer taraftan, QbD'nin uygulanmasında zorunlu görülmeyen ancak birçok üstünlüğe sahip bir araç olan PAT ile azaltılan kontroller ve daha az sorunlu seri üretilmesi gibi faktörlerle maliyetler düşmekte ve daha yüksek yatırım getirisi elde edilmektedir (Beg et al., 2019).

Proses ile ilgili derinlemesine bilgi sahibi olunması ve tüm güncellemeleri destekleyecek verilerin halihazırda mevcut olması sayesinde üretim prosesinde yapılan değişiklikleri takip eden resmi dokümanların güncellenmesinde sadece küçük varyasyonlarla sağlanabilmektedir (Zacché & Andersson, 2019). Geleneksel yaklaşımdan farklı olarak, ürün ve proses iyi anlaşılmış olduğundan, yapılacak değişikliklerin kabul edilebilir olup olmadığına kolayca karar verilebilmektedir. Geleneksel yaklaşımda ise değişikliklerin sebep olabileceği etkiler bilinmediğinden yapılacak değişikliklere karar verilip uygulanması, masraflı yatırımlar ve zaman kaybına sebep olan yeni verilerin toplanmasını gerektiren kapsamlı ruhsat varyasyonlarına neden olabilmektedir. Bu doğrultuda QbD yaklaşımında sürekli gelişime açık ve esnek proses anlayışı benimsenirken, geleneksel yaklaşımda ise değişikliğe açık olmayan daha sabit bir proses anlayışının hakim olduğu görülmektedir.

QbD yaklaşımını uygulayan 12 farklı ilaç firması ile yürütülen bir araştırmada, firmaların "proses ve ürün anlayışını geliştirmesi, ürün geliştirme sürecine daha sistematik bir bakış açısı sağlaması, hasta güvenliliğinin artırılması, ürün geliştirme ve üretim verimliliğini artırması" ile ilgili beklentilerinin bu yaklaşımın uygulanılmasıyla karşılanabildiği gösterilmiştir (Kourti & Davis, 2012). Firmaların QbD yaklaşımını, sadece geliştirilen ürünlerden maddi kazanç sağlanması ve ruhsatlandırma başvurularında esneklik imkanı tanıması yönüyle değil aynı zamanda Resmi Otorite beklentilerinden bağımsız olarak, doğru ve faydalı buldukları için uyguladıkları ortaya çıkmıştır.

Resmi Otorite açısından bakıldığında da, ilaç geliştirme ve üretim süreçlerini uygulayanlar, QbD yaklaşımını benimsemeleri yönünde resmi otoriteler tarafından teşvik edilmektedir (Kannissery Pramod et al., 2016).

Tasarımla Kalite yaklaşımının benimsenmesi ile ruhsatlandırma sürecinde sunulan kapsamlı dokümanlar sayesinde tesisin proseste yaptığı değişikliklerle ilgili daha esnek bir başvuru ve denetim süreci yaşandığı da görülmektedir (Sangshetti et al., 2017). Tesis tarafından hazırlanan proses validasyonu ve elde edilen seriler arası tutarlılık ile sağlanan güven sayesinde ruhsatlandırma sonrası otorite talepleri azalmakta, ilacın pazara hızlı şekilde ulaştırılması ve geri çekmelerin azaltılması ile ilacın pazarda bulunur olması sağlanmaktadır.

Ruhsatlandırma sürecinde sunulan bilgilerin yönetimi ile ilgili rehberlik oluşturması amacıyla; QbD'nin anlatıldığı ICH Q8 ile başlayan yeni farmasötik kalite yaklaşımının bir sonraki önemli adımı olarak 20/11/2019 tarihinde ICH Q12 "ICH Q12 Technical and Regulatory Considerations for Pharmaceutical Product Lifecycle Management Step 4" yayımlanmıştır. İlerleyen yıllarda ICH Q12 kılavuzunun resmi otoriteler tarafından benimsenmesi, hem resmi otoritelerin hem de ruhsat sahiplerinin iş yükünün azalmasına, aynı zamanda ruhsatlandırma öncesi ve sonrası süreçlerin kısalmasına da katkı sağlayacaktır.

4. SONUÇ

Farmasötik geliştirmenin amacı, ürünün hedeflenen performansını tutarlı bir şekilde sağlamak için kaliteli bir ürün ve üretim prosesi tasarlamaktır. Bu tutarlılığa ulaşmanın başlıca yolu hastaların ihtiyaçlarını karşılayacak bir ürün için uygun şekilde kontrol edilen kapsamlı bir proses kavrayışına sahip bir üretim prosesi oluşturmaktır.

Bu derlemede ilaç üretiminde istenilen kaliteye ulaşmak için önemli olan, üretimin proses validasyonu ile ilgili ulusal ve uluslararası kılavuzlar doğrultusunda güncel yaklaşımlar üzerinde durulmuştur. Bu yaklaşımlar geleneksel proses validasyonuna alternatif olarak sunulan kesintisiz proses doğrulaması be bunun uygulanmasında kullanılabilen bir yöntem olan tasarımla kalite ve sağlayabileceği üstünlükler üzerinde durulmuştur. Tasarımla kalite, stratejisi tasarımla kalite yaklaşımı üzerine kurulan ICH Q8, Q9 ve Q10 kılavuzları ile hayata ve uygulamaya geçmiştir.

Tasarımla Kalite yaklaşımının uygulanması; Hedef Ürün Profillerinin tanımlanması ve gerekçelendirilmesi, ürün ve proses anlayışı kazandırması, uygun kontrol stratejileri ile bir kontrol durumunun sağlanması, risk yönetiminin daha etkin şekilde kullanılması ve aynı zamanda düzenleyici otoriteye yapılacak başvuruların yükünü azaltması ile hem hasta hem üretici hem de düzenleyici otorite açısından fayda sağlamaktadır.

Ülkemizde gerçekleştirilen uygulamaya bakıldığında; PIC/S üyesi bir ilaç otoritesi olarak; Türkiye'de bulunan ilaç üretim tesislerinin denetimlerinin sıklığı risk bazlı olarak belirlenmektedir. Risk parametreleri arasında ilgili tesisin önceki denetimlerinde tespit edilen eksikliklerin seviyesi ve sayısı ile belirlenen İİU seviyesi önemli bir rol oynamaktadır. Dolayısıyla tesislerin İİU seviyesinin artması denetim sıklığını azaltmaktadır. Otorite denetimlerinin sıklığının azalması ile hem üretici hem düzenleyici otorite açısından iş gücü kaybının önüne geçilmesini sağlanacaktır. Bununla birlikte, QbD yaklaşımının Ülkemizde üretim yapan tesislerde uygulanmaya başlanması ile daha sağlam bir proses anlayışı kazanılması ve tutarlı seriler üretilmesi sayesinde hem tesisin ilaç otoritesi nezdindeki İİU seviyesi artacak hem de geri çekmelerin azalması maddi kayıpların önüne geçilecek ve yüksek kalitede ilaçların piyasada bulunabilir olması sağlanacaktır. Düzenleyici otoriteler ve dünya ilaç sektöründe de benimsenen ve her geçen gün yaygınlığı artmakta olan QbD yaklaşımı ülkemizde de benimsenmeye başlanmış olup bu teknoloji ile üretim gerçekleştiren ilaç firmaları bulunmaktadır. Bu yaklaşıma sahip tesislerin başvuruları TİTCK tarafından değerlendirilmekte ve denetlenmektedir.

Geleneksel proses validasyonu ile işletilen bir proseste testlere ve denetime dayalı bir kontrol stratejisi mevcut iken ve bitmiş ürün de yapılan örneklem testler ile daha yüzeysel bir proses anlayışı hakim iken QbD yaklaşımının uygulanması ile değişkenlerin (parametrelerin) ürüne ve prosese etkisi geniş çerçevede, daha detaylı bir bakış açısıyla değerlendirildiği için daha derin bir proses anlayışına sahip olunmakta, daha ileri yaklaşım olan gerçek zamanlı serbest bırakma, çevrimiçi test etme ve riske dayalı bir yaklaşım uygulanabilmektedir. Geleneksel yaklaşımda örneklem olarak seçilmiş numunelerde gerçekleştirilen testler bütün bir seriyi temsil ediyorken QbD yaklaşımında ise kontrol stratejisi serinin tamamına uygulanmaktadır. Bitmiş üründe gerçekleştirilen kalite kontrol testlerinin yerine QbD yaklaşımının uygulandığı durumda bitmiş ürün testleri gerçekleştirilmeyebileceğinden dolayı analiz için gereken personel, ekipman, reaktif ve referans çözelti kullanımları ve işgücü gibi birçok alanda üstünlük sağlayabilmektedir. Tüm bu faydalara ilaveten, ilaç endüstrisinde ve uygulanan teknolojilerde gelişmelerle birlikte, ICH Q8, Q9 ve Q11 vizyonunun devamı niteliğindeki yeni yaklaşımların getirildiği ICH Q12 Yaşam döngüsü Yönetimi ve ICH Q13 Etkin madde ve İlaçların Sürekli Üretimi kılavuzlarının hayata geçebilmesi için ilk adım olarak QbD'nin benimsenmesi ve uygulanması önemlidir. Ancak proses validasyonunda güncel yaklaşımların uygulanması önemli bir kültür değişikliği gerektirdiğinden düzenleyici kurumlar, endüstri ve

akademisyenler, çok disiplinli bir yaklaşımda birlikte çalışması gerekmektedir.

YAZAR KATKI ORANI BEYANI

Yazarların katkı oranı eşittir

ÇIKAR ÇATIŞMASI

Yazarlar herhangi bir çıkar çatışması olmadığını beyan eder.

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REVIEW ARTICLES

Tip 2 Diyabet Tedavisinde Kullanılan Exendin-4 ve Exendin-4'ün Alternatif Uygulama Yolları İçin Güncel Yaklaşımlar

Merve ÇELİK TEKELİ[*°], Yeşim AKTAŞ[**], Nevin ÇELEBİ[***]

Exendin-4 Used in the Treatment of Type 2 Diabetes and Current Approaches for Alternative Administration Routes of Exendin-4

SUMMARY

Diabetes is a chronic disease due to impaired glucose metabolism and usually presents with uncontrolled hyperglycemia or persistently high blood sugar levels. According to the tenth edition of the International Diabetes Federation, 10% of global health expenditures ($ 966 billion) are spent on diabetes. In Turkey, the prevalence of diabetes is 15%, showing the fastest increase among European countries. Type 2 diabetes is associated with abnormal insulin secretion or chronic insulin resistance, causing desensitization of the glucose uptake cells to insulin activity. Incretin-based therapies have come to the fore in the treatment of Type 2 diabetes in recent years. Exendin-4, which is widely used in incretin-based therapies, binds to GLP-1 receptors with high affinity, causing glucose-dependent insulin secretion in the body, delaying gastric emptying, suppressing glucagon release and appetite. Also, exendin-4 increases cell proliferation and inhibits apoptotic pathways in β-cells. Commercial products of exendin-4 are Byetta® which is administered twice daily and long-acting Byderuon™ which is administered once weekly via parenteral route. The presence of drawbacks of parenteral administration such as problems in patient compliance and feeling of pain due to injection led researchers to search alternative administration routes such as oral, pulmonary, transdermal, ocular, nasal, vaginal and rectal routes. In this review, exendin-4's properties, mechanism of action, therapeutic efficacy, new approaches and studies on alternative delivery routes of exendin-4 are mentioned.

Key Words: *exendin-4, diabetes, oral route, pulmonary route, transdermal route, nanocarriers*

Tip 2 Diyabet Tedavisinde Kullanılan Exendin-4 ve Exendin-4'ün Alternatif Uygulama Yolları İçin Güncel Yaklaşımlar

ÖZ

Diyabet, bozulmuş glikoz metabolizması ile ilişkili kronik bir hastalıktır ve genellikle kontrolsüz hiperglisemi veya sürekli yüksek kan şekeri seviyeleri ile kendini göstermektedir. Uluslararası Diyabet Federasyonu verilerine göre küresel sağlık harcamalarının %10'unun (966 milyar Amerikan Doları) diyabete harcandığı belirtilmiştir. Türkiye'de ise diyabet prevalansı %15 olarak Avrupa ülkeleri içinde en hızlı artışı göstermektedir. Tip 2 diyabet, anormal insülin sekresyonu veya kronik insülin direnci ile ilişkilidir ve glikoz alım hücrelerinin insülin etkisine karşı duyarsızlaşmasına neden olur. Son yıllarda Tip 2 diyabet tedavisinde inkretin bazlı tedaviler öne çıkmıştır. İnkretin bazlı tedavilerde yaygın kullanılan exendin-4, GLP-1 reseptörlerine yüksek afinitede bağlanarak vücutta glikoz bağımlı insülin salgılanmasını, gastrik boşalmanın geciktirilmesini, glukagon salımının ve iştahın baskılanmasını sağlamaktadır. Exendin-4, hücre proliferasyonunu artırmakta ve β-hücrelerinde apoptotik yolları inhibe etmektedir. Exendin-4'ün ticari ürünleri parenteral yolla günde iki kez uygulanan Byetta® ve haftada bir kez uygulanan uzun etkili Byderuon™'dur. Parenteral yolla uygulamanın hasta uyuncunda problemler ve enjeksiyonda ağrı hissi gibi sakıncalarının bulunması araştırmacıları oral, pulmoner, transdermal, oküler, nazal, vajinal, rektal yollar gibi alternatif veriliş yolları üzerine yöneltmiştir. Bu derlemede, exendin-4'ün özellikleri, etki mekanizması, terapötik etkinliği ve exendin-4'ün alternatif veriliş yollarına yönelik yeni yaklaşımlar ve yapılan çalışmalar verilmiş ve tartışılmıştır.

Anahtar kelimeler: *exendin-4, diyabet, oral yol, pulmoner yol, transdermal yol, nanotaşıyıcılar*

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GİRİŞ

Diyabet yüksek kan glikoz seviyesiyle karakterize edilen akut ve kronik komplikasyonların eşlik ettiği metabolik bir hastalıktır (WHO, 2006). Her yıl 1,6 milyon ölüm diyabetle doğrudan ilişkilendirilmektedir (Saeedi ve ark., 2019). Uluslararası Diyabet Federasyonu verilerine göre ise 2021'de 6,7 milyon ölümün diyabete bağlı olduğu belirtilmiştir (International Diabetes Federation, 2021).

Tip 2 diyabet tedavisinde insülin sıklıkla kullanılmaktadır. Ancak parenteral yolla uygulanan insülin dozunun doğru ayarlanamaması hipoglisemiye yol açmaktadır. Ayrıca iğne fobisi ve lipodistrofi gibi nedenlerden dolayı hasta uyuncu açısından da parenteral insülin uygulaması sorun teşkil etmektedir. İnsülin tedavisi dışında Tip 2 diyabet tedavisinde oral antihiperglisemik ilaçlar kullanılmaktadır. Bu ilaçlar insülin duyarlılaştırıcı, insülin salgılatıcı, insülinomimetik (inkretin bazlı) ilaçlar ve alfa glukozidaz inhibitörleri olarak dört grupta sınıflandırılmaktadır (Marín-Peñalver ve ark., 2016).

Exendin-4 pankreatik GLP-1 reseptörlerine yüksek afiniteyle bağlanarak antidiyabetik etki gösteren GLP-1 reseptör agonisti grubunda yer alan peptit yapısında bir moleküldür. 2005'de FDA tarafından onaylanan exendin-4'ün Byetta® ticari ismiyle 5 µg ve 10 µg'lık subkütan formları mevcuttur. Exendin-4'ün subkütan formu Byetta® pankreastaki beta hücrelerinden insülin sekresyonunu artırırken aynı zamanda glukagon sekresyonunu da baskılamaktadır. Bu sayede exendin-4, kan glikoz konsantrasyonunu düzenlemekte etkin bir rol oynamaktadır (Gao ve Jusko, 2012). Exendin-4, insülin ve diğer antidiyabetik ajanların aksine hipoglisemiye yol açmaksızın kan glikoz seviyelerini düzenlemekte ve bu sayede endojen insüline karşı bozulmuş biyolojik yanıt olarak tanımlanan insülin direnci oluşumunu da önlemektedir (Chakraborti, 2010).

Son yıllarda biyoteknolojideki gelişmelerle exendin-4'ün haftada bir kez uygulanabilen uzun etkili formülasyonu Byderuon™ geliştirilmiştir. Parenteral yol dışında exendin-4'ün alternatif veriliş yolları üzerinde çalışmalar özellikle oral, pulmoner ve transdermal yol üzerine yoğunlaşmıştır. Bu derlemede, exendin-4'ün özellikleri, etki mekanizması, terapötik etkinliği ve farklı uygulama yollarına yönelik yeni yaklaşımların yer aldığı çalışmalar anlatılacaktır.

GENEL BİLGİLER

Exendin-4'ün Diyabet Tedavisinde Kullanımı

Diabetes mellitus, insülin etkisi, insülin salımı ya da her ikisinde de bozukluk sebebiyle ortaya çıkan hiperglisemiyle ilişkili akut ve kronik komplikasyonların eşlik ettiği metabolik bir hastalık olarak tanımlanmaktadır (Punthakee ve ark., 2018). Tip 2 diyabet dünyada en sık rastlanan diyabet tipi (%90) olup, insülin sekresyon bozukluğu, dokularda insülinin kullanılamaması (insülin direnci) ve obeziteyle ilişkili bir hastalıktır (TEMD, 2019).

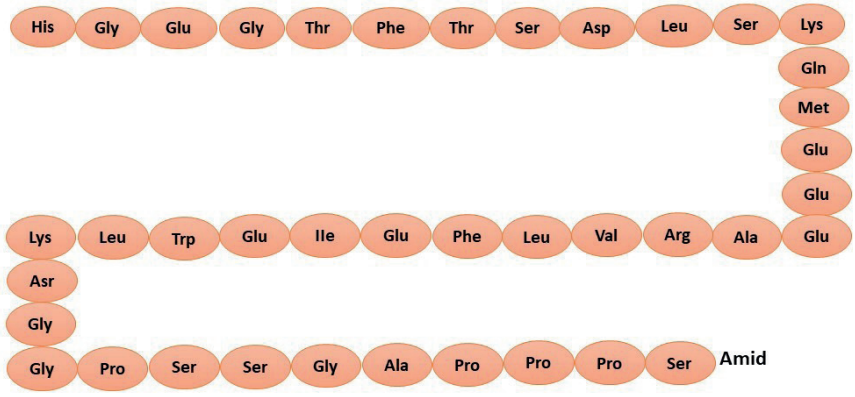
Uluslararası Diyabet Federasyonu Diyabet Atlası onuncu baskı verilerine göre 2021'de, 10 kişiden birinde (537 milyon) diyabet tespit edilmiştir. Bu sayının 2045'de 784 milyona ulaşacağı tahmin edilmektedir. Küresel sağlık harcamalarının %10'unun (966 milyar ABD Doları) diyabete harcandığı belirtilmiştir (International Diabetes Federation, 2021). Türkiye'de ise 2020'de diyabet prevalansı %15 olup, sağlık harcamalarının %10'u diyabet tanı ve tedavisine harcanmıştır (T.C. Çalışma ve Sosyal Güvenlik Bakanlığı, 2021).

Diyabet tedavisi; temel olarak diyetin düzenlenmesi ve egzersiz, Tip 2 diyabetlilerin ideal ağırlığına döndürülmesi, insülin uygulanması ve Tip 2 diyabetlilere oral antidiyabetik ilaç verilmesini içermektedir. Diyabet tedavisinde hedef, hipergliseminin mümkün olduğunca normal seviyeye (açlık kan glikoz seviyesi<100 mg/dL) düşürülmesi ve normale yakın tutulmasıdır. (Kayaalp ve Gürlek, 2009). Oral yol bilindiği üzere hasta uyuncu açısından en fazla tercih edilen uygulama yoludur. Oral antidiyabetik ilaçlar insülin duyarlılaştırıcılar, insülin salgılatıcılar, insülinomimetik (inkretin bazlı) ilaçlar ve alfa glukozidaz inhibitörleri olarak dört grupta sınıflandırılmaktadır

(Luna ve Feinglos, 2001). Tip 2 diyabette inkretin hormonların seviyesi ve/veya etkisi azalmakta ve glukagon sekresyonu inhibe edilememektedir. İnkretin bazlı ilaçlar, inkretin hormonlarını taklit eden glukagon benzeri peptit-1 reseptör agonistleri (GLP-1A) ya da inkretinlerin parçalanmasını inhibe eden dipeptidil peptidaz-4 inhibitörleri (DPP4-İ) olarak sınıflandırılabilir. GLP-1 ve glikoza-bağımlı insülinotropik peptit (GIP) intestinal bölgeden salınan inkretinler olarak adlandırılan insülin salımının düzenlenmesinden sorumlu hormonlardır (Kim ve Egan, 2008). Glikoz-bağımlı etkilerinden dolayı hipoglisemiye yol açmamaktadırlar. İnkretin mimetikler pankreas beta hücrelerinden insülin salgılanmasını artırırlar ve glukagon salgılanmasını azaltırlar. Midenin boşalmasını geciktirerek besinlerin sindirimini ve barsaktan emilimini yavaşlatmaktadırlar. Ayrıca iştahı azalttıkları için besin alımını azaltma ve kilo kaybı yapma potansiyelleri vardır. GLP-1A grubunda exendin-4 (eksenatid) ve liraglutid yer almaktadır. DPP4-İ grubunda ise linagliptin, siltagliptin ve vildagliptin yer almaktadır (Levetan, 2007; Katzung, 2017).

Exendin-4'ün Yapısı ve Özellikleri

Exendin-4, Gilia monster (*Heloderma suspectum*) adı verilen bir tür sürüngenin tükrüğünden izole edilen GLP-1 ile aminoasit dizilimine %50'den fazla benzerlik gösteren ve 39 aminoasitten oluşan doğada bulunan en iyi GLP-1 agonisti molekülüdür. GLP-1 molekülündeki 2. aminoasit pozisyonundaki Ala, DPP-4 enziminin exendin-4 molekülünde parçalayıcı etkisini önlemek amacıyla Gly ile değiştirilmiştir. Bu sayede exendin-4 enzim etkisine karşı korunmaktadır. Exendin-4, GLP-1 reseptörlerine yüksek afinitede bağlanarak vücutta glikoz bağımlı insülin salgılanmasının yanısıra gastrik boşalmanın geciktirilmesini, glukagon salımının ve iştahın baskılanmasını sağlamaktadır (Ükinç ve ark., 2007; Kayaalp ve Gürlek, 2009). Exendin-4'ün aminoasit dizilimi Şekil 1'de gösterilmektedir.



Şekil 1. Exendin-4'ün aminoasit dizilimi (Chen ve Drucker, 1997'den adapte edilmiştir).

Exendin-4'ün molekül ağırlığı 4186,57 Da, izoelektrik noktası 4,96 olup (Kim ve ark., 2013), çözünürlüğü DMSO'da 20 °C'de yaklaşık 1 mg/mL bulunmuştur (Bachhav ve Kalia, 2011). Exendin-4'ün permeabilitesinin araştırıldığı bir çalışmada; Madin Darby Canine Kidney (MDCK) hücrelerinden exendin-4'ün geçişi incelenmiş, permeabilite katsayısı (P_{app}) 1×10^{-7} cm^2/s olarak bulunmuştur. Exendin-4'ün intestinal membrandan geçişinin zayıf olduğunu ve geçişin pasif parasellüler yolla olduğu belirtilmiştir (Wang ve ark., 2014). Exendin-4'ün, Tip 2 diyabetli hastalara subkütan enjeksiyonla uygulanmasını takiben plazma doruk konsantrasyonuna (C_{maks}) yaklaşık iki saatte ulaştığı belirtilmiştir. Exendin-4 subkütan yoldan 10 µg dozda verildiğinde C_{maks} 211 pg/mL ve ortalama eğri altında kalan alan (AUC) ise 1036 pg.s/

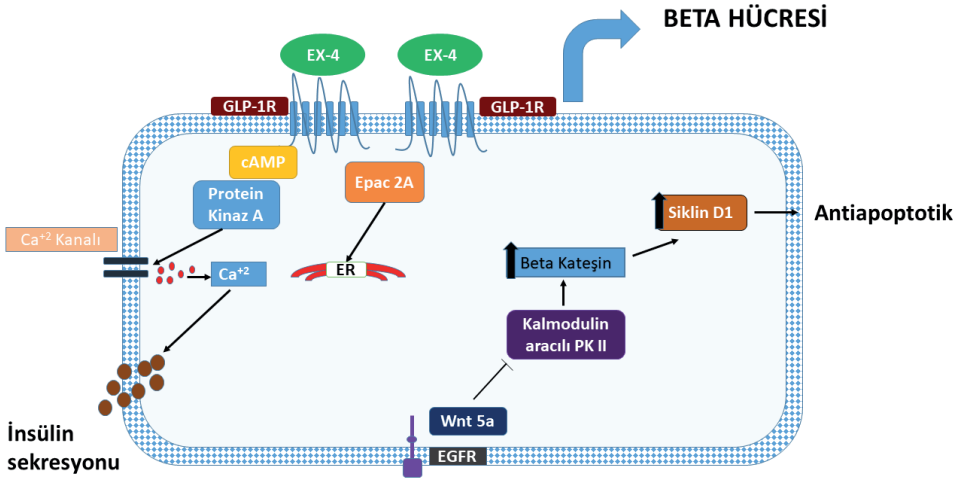
mL olarak bulunmuştur. Exendin-4, sırasıyla 5 ve 10 µg terapötik doz aralığında uygulandığında AUC değerinin verilen dozla orantılı olarak arttığı belirtilmiştir. Subkutan tek doz uygulandığında ortalama sanal dağılım hacminin 28,3 L, ortalama klirensin 9,1 L/s, ve ortalama terminal yarı ömrünün 2,4 saat olduğu belirtilmiştir (Malone ve ark., 2009). Exendin-4'ün Byetta® ticari ismiyle 5 µg ve 10 µg'lık subkütan formları mevcuttur. Byetta® FDA 2005 yılında, Avrupa Birliği'nde 2006 yılında, Türkiye'de ise 2008 yılında Tip 2 diyabetin tedavisi için ruhsat almıştır. Sunulan vaka raporlarında ve yapılan çalışmalarda Byetta®'nın pankreatit riskini artırdığı belirtilmektedir (Bain ve ark., 2008). Exendin-4'ün ülkemizde henüz ruhsatlı olmayan ticari ürünü Bydureon® uzatılmış salım göstermektedir. Bydureon®, haftada bir subkütan injeksiyon şeklinde kullanılmak üzere FDA tarafından 2012'de onaylanarak Amerika'da ve Avrupa'da satışa sunulmuştur (Kayaalp ve Gürlek, 2009; TEMD, 2019). Sık görülen advers etkisi tedavi ilerledikçe şiddeti azalan mide bulantısıdır. Ayrıca, baş ağrısı, diyare, akut pankreatit, immünojenisite görüldüğü belirtilmiştir (Tucker ve ark., 2013). Byetta® ve Bydureon®' un yanısıra exendin-4 içeren implante edilebilen ozmotik pompa (ITCA 650) için FDA'ya ruhsat başvurusunda bulunulmuş ancak klinik araştırmalarda gözlenen akut böbrek hasarı sebebiyle başvuru reddedilmiştir (Bertsch ve McKeirnan, 2018; FDA, 2021).

Exendin-4'ün Etki Mekanizması

Bağırsak L-hücreleri tarafından salgılanan endojen bir peptit hormonu olan GLP-1 inkretin etkisi göstermektedir. GLP - 1 reseptörünün endojen GLP - 1 peptidi tarafından aktivasyonu, protein kinaz A ve siklik adenin mono fosfat (cAMP) tarafından indüklenen değiştirici protein (Epac) ilişkili cAMP'ye bağımlı ikincil haberci yolaklarını başlatan adenilil siklazı içermektedir. GLP - 1 reseptörünün aktivasyonunu takiben, insülin salgılanmasını kolaylaştırmak için insülin içeren veziküllerin endoplazmik retikulumun (ER) Ca^{+2} ile indüklenen ekzositozunu tetiklemektedir. GLP-1 reseptörünün aktivasyonu, glukagon sekresyonunun inhibisyonu ve pankreas beta hücrelerinden glikoza bağımlı insülin salgılanmasını sağlamaktadır. Özellikle gıda alımı veya parenteral glikoz enjeksiyonundan sonra, plazma glikoz seviyeleri yüksek olduğunda hücre içine glikoz alımını kolaylaşmaktadır. Exendin-4, GLP-1 reseptörünü aktive etmede endojen GLP-1 kadar güçlüdür. Plazma glikoz seviyeleri yüksek olduğunda GLP-1 reseptörüne bağlanıp aktive ederek insülinotropik etkisini ortaya çıkarmaktadır (Yap ve ark., 2019). Exendin-4, Tip 2 diyabet tedavisinde sıklıkla kullanılsa da etki mekanizmaları açısından benzer moleküler mekanizmalara sahip nörodejeneratif ve kardiyovasküler bozuklukların da tedavisinde umut verici olmuştur (Boztaş ve ark., 2017). Öte yandan exendin-4'in etkisi, epidermal büyüme faktörü reseptörü (EGFR) aracılı Wnt5a geni aracılığıyla siklin D1'in ekspresyonunu da artırarak hücre proliferasyonunu artırmakta ve β-hücrelerinde apoptotik yolları inhibe etmektedir (Lee ve Jun, 2014). Exendin-4'ün insülinotropik ve antiapoptotik etki mekanizması Şekil 2'de gösterilmektedir.

Exendin-4'ün Uygulama Yolları

Terapötik peptit/proteinler genellikle parenteral yolla uygulanmaktadır. Ancak parenteral yolla uygulamanın zayıf hasta uyuncu ve enjeksiyonda ağrı hissi gibi sakıncalarının bulunması oral, pulmoner, transdermal, oküler, nazal, vajinal, rektal yollar gibi alternatif veriliş yolları üzerine araştırmacıları yöneltmiştir (Ibraheem ve ark., 2014). Exendin-4'ün alternatif veriliş yollarıyla normoglisemik (diyabetik olmayan) sıçanlara uygulanması sonucu elde edilen farmakokinetik parametreleri karşılaştırılmıştır (Gedulin ve ark., 2008). Exendin-4'ün veriliş yollarına göre sıçanlarda saptanan farmakokinetik parametreleri Tablo 1'de verilmiştir.



Şekil 2. Exendin-4'ün insülinotropik ve antiapoptotik etki mekanizması (Yap ve ark., 2019'dan adapte edilmiştir).

Tablo 1. Exendin-4'ün veriliş yollarına göre farmakokinetik parametreleri (Gedulin ve ark., 2008).

Veriliş Yolu	k_a (dk^{-1})	k_e (dk^{-1})	AUC_{480}	Doz (µg)	AUC/doz (pM dk/µg)	Bağıl Biyo-yararlanım (%)	C_{maks} (pM / µg)
İntraduodenal	0,490	0,045	2.895	1000	2,890	0,005	0,115
Sublingual	0,210	0,011	42.737	210	203,500	0,370	2,110
İntranazal	0,150	0,017	92.100	100	921,000	1,680	16,060
Aerosol	0,410	0,017	100.285	2000	50,100	0,092	0,710
İntravenöz	0,091	0,017	114.820	210	54.600,000	100,000	3.757,000
Subkütan	0,099	0,004	7.080.240	210	33.715,000	61,700	134,300
İntratrakeal	0,120	0,014	1.556.000	210	7.410,000	13,600	327,100

Tablo 1'de görüldüğü üzere intravenöz uygulama ile karşılaştırıldığında exendin-4'ün subkütan bağıl biyoyararlanımı %61,7 olarak bulunmuştur. Ancak intravenöz uygulama yollarıyla karşılaştırıldığında diğer uygulama yollarında bağıl biyoyararlanım oldukça düşük bulunmuştur. Exendin-4'ün subkütan uygulanan preparatlarının etkinliğini iyileştirmek ve uzun etkili olmasını sağlamak için araştırmalar devam etmektedir. Ayrıca exendin-4'ün parenteral yol dışında alternatif veriliş yollarıyla ilgili de farklı yaklaşımlar bulunmaktadır. Exendin-4'ün parenteral yol ve alternatif veriliş yolu ile ilgili çalışmalar aşağıda verilen başlıklar altında değerlendirilmiştir.

Exendin-4'ün Parenteral Yolla Uygulanması

Exendin-4'ün ticari ürünleri günde iki kez uygulanan Byetta® ve haftada bir kez uygulanan uzun etkili Byderuon™ parenteral yolla uygulanmaktadır. Bydureon™, Byetta®'ya göre bazı avantajlara sahiptir. Byderuon™, exendin-4 içeren mikroküreler içermektedir ve mikroküreler exendin-4'ün uzun etki göstermesini sağlamıştır. Mikroküreler, boyutları 1–1000 µm arasında değişen küresel parçacıklar olarak tanımlanmaktadır (Saralidze ve ark, 2010). Biyoparçalanabilen polimerik mikroküreler kontrollü salım sağlanmasında ve hedeflendirmede sıklıkla kullanılmaktadır (Freiberg ve Zhu, 2004). Bydureon™ biyouyumlu ve

biyoparçalanabilen polilaktik-ko-glikolik asit (PLGA) mikroküreleri içermektedir. Bununla birlikte, Bydureon™ 'nun kısa ürün bilgilerinde (KÜB), ciddi vakalarda cerrahi müdahale gerektirebilen, nodüller ya da enjeksiyon bölgesinde apse, selülit ve nekroz gibi reaksiyonlar bildirilmiştir (FDA, 2018). Bydureon™'da exendin-4 içeren PLGA mikroküreleri, salım profilinde gecikme fazı göstermektedir. Yüzeydeki gevşek bağlı exendin-4 nedeniyle ilk 2 gün içinde ani salımdan sonra, salım yedi hafta sürmektedir. Bu gecikme fazının varlığı, exendin-4'ün zayıf etkinlik göstermesine yol açmaktadır (Cai ve ark., 2013). Bu durum araştırmacıları Bydureon™ mikroküreden daha iyi bir formülasyon tasarlama üzerine yöneltmiştir. Exendin-4'ün kontrollü salım sağlayan formülasyonları üzerine araştırmalar devam etmektedir. Parenteral yolla verilmek üzere hazırlanan kontrollü salım sağlayan exendin-4 içeren ilaç taşıyıcı sistemler Tablo 2'de gösterilmiştir.

Tablo 2. Parenteral yolla uygulanmak üzere hazırlanan exendin-4 içeren ilaç taşıyıcı sistemler.

İlaç Taşıyıcı Sistem	Sonuç	Kaynak
PLGA mikroküre	Mikrokürelerin 21 gün boyunca salım gösterdiği belirtilmiştir. Günde iki kez exendin-4 uygulamasıyla karşılaştırıldığında, iki haftada bir uygulanan mikrokürelerin, açlık kan şekerini ve HbA1c konsantrasyonlarını daha etkili bir şekilde düşürdüğü belirtilmiştir.	(Kwak ve ark., 2009)
Pullulan polisakkariti (asetilasyon derecesi 0,8, 1,5 ve 2,3) içeren mikroküre	Mikrokürelerin partikül boyutu 35-110 μm aralığında bulunmuştur. Daha yüksek derecede asetilasyonun (2 ve 3), daha yüksek enkapsülasyon etkinliği (%90,3) gösterdiği bulunmuştur. Exendin-4'ün moleküler ağırlığında herhangi bir kayıp olmaması, exendin-4'ün polisakkarit mikrokürelerde iyi korunduğunu göstermiştir. Bununla beraber, immünolojik çalışmalar polisakkarit mikrokürelerin inflamasyona yol açtığını göstermiştir.	(Yang ve ark., 2009)
PLGA mikroküre	Mikrokürelerin 18 gün boyunca salım gösterdiği belirtilmiştir. PEG-exendin-4 içeren PLGA mikrokürelerinin exendin-4'ün stabilitesini artırdığı belirtilmiştir. Diyabetik farelerde yapılan *in vivo* çalışmada PEG-exendin-4 içeren PLGA mikrokürelerinin diyabetik farelerde kan glikoz seviyesini 12 güne kadar normal düzeyde tuttuğu belirtilmiştir.	(Lim ve ark., 2015)
Nanopartikül/ Pluronic F-127 jel	Diyabetik farelerde yapılan *in vivo* çalışmada exendin-4, exendin-4 içeren çok tabakalı nanopartiküller, exendin-4 içeren çok tabakalı nanopartiküller/Pluronic F-127 karışımının sc uygulaması sonucu 10. saatte kan glikoz seviyesinin sırasıyla %26,9, %60,8, %74,3 azaldığı belirtilmiştir. Ayrıca çok tabakalı nanopartiküller/ Pluronic F-127 karışımının uzatılmış salım göstermesiyle diyabetik farelerde kan glikoz seviyesinin 3 gün boyunca normoglisemik düzeyde olduğu gösterilmiştir.	(Oh ve ark., 2014)

Exendin-4'ün Oral Yolla Uygulanması

Oral yolun hasta uyuncu ve kullanım kolaylığı açısından en çok tercih edilen uygulama yolu olması araştırmacıları peptit/proteinlerin oral yolla verilmesi üzerine yoğunlaştırmıştır. Ancak peptit/proteinlerin gastrik ortamın pH'sı ve proteolitik enzimlerin varlığı sebebiyle stabilite problemleri vardır ve oral biyoyararlanımları düşüktür. Bu nedenle peptit/proteinlerin oral biyoyararlanımlarını artırmak için kimyasal modifikasyon, absorbsiyon artırıcı maddelerin ilavesi, proteaz inhibitörlerinin formülasyona eklenmesi ve ilaç taşıyıcı sistemlerin geliştirilmesi gibi çeşitli yaklaşımlar geliştirilmiştir (Tan ve ark. 2010, Renukuntla ve ark. 2013). Bu yaklaşımların ana amacı, gastrointestinal bariyerlerin sınırlayıcı etkilerinin üstesinden gelmek ve oral biyoyararlanımı artırmaktır. Bu yaklaşımlar tek başına veya kombinasyon halinde kullanılmaktadır. Exendin-4'ün kimyasal modifikasyonu ile yapılan bir çalışmada exendin-4'ün biotinizasyonunun exendin-4'ün oral antidiyabetik etkinliğine etkisi araştırılmıştır. Biotinilasyon işleminin özellikle biotinle konjuge exendin-4'ün (DB-Ex-4) lizin kalıntılarını bloke ederek, tripsin enzimi ve intestinal sıvıda exendin-4'ün proteolitik stabilitesini sırasıyla 8,4 ve 9,0 kat artırdığı bulunmuştur. DB-Ex-4'ün 0,1, 1, 10 μg/kg dozda diyabetik farelere oral uygulanması sonucu doza bağlı artan hipoglisemik etki gözlenmiştir. Konjuge haldeki exendin-4 serbest exendin-4'e göre 5,3 kat fazla hipoglisemik etki gösterdiği ve bağıl bi-

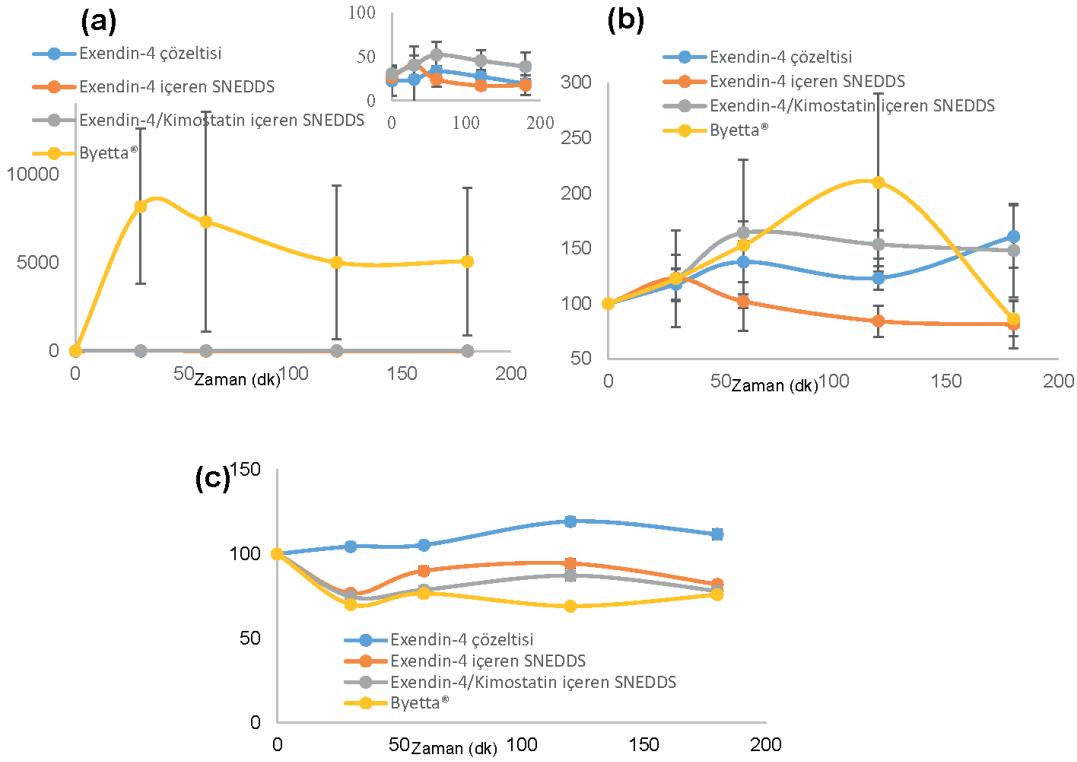
yoyararlanım %3,95 bulunmuştur (Jin ve ark., 2009).

Exendin-4'ün oral biyoyararlanımını artırmak için ilaç-polimer konjugatlarından da yararlanılmıştır. Bir çalışmada, oral yoldan verildiğinde hipoglisemik etkinliğinin artırılması için exendin-4'ün düşük molekül ağırlıklı kitosanla (LMWC) konjugasyonu yapılmıştır. Diyabetik sıçanlarda yapılan intraperitoneal olarak verilen glikoz tolerans testiyle; oral verilen formülasyonun kan glikoz seviyelerindeki değişiklikler zamana karşı ölçüldüğünde, serbest exendin-4 ihmal edilebilir düzeyde hipoglisemik etki gösterirken, LMWC-exendin-4 4 µg/kg ve 40 µg/kg oral uygulandıklarında glikoz seviyesini sırasıyla %22,90 ± 2,0 ve %41,07±4,7 oranında düşürmüşlerdir. LMWC-exendin-4 konjugatının oral uygulamadan sonra *in vivo* hipoglisemik etkinliği, farmakokinetik verilerle uyumlu bulunmuştur. Bu durumun düşük molekül ağırlıklı kitosanın mukoadeziv özelliğine ve epitel hücreleri arasındaki sıkı bağlantıları açma yeteneğine bağlı olduğu düşünülmektedir (Ahn ve ark., 2013).

Son yıllarda peptit/protenlerin oral biyoyararlanımlarını artırmak için nanotaşıyıcı sistemlerden sıklıkla yararlanılmaktadır. Exendin-4'ün oral yolla verilmesinde polimerik nanotaşıyıcılar (polimerik nanokapsüller, polimerik nanopartiküller) ve lipit bazlı nanotaşıyıcılardan (kendiliğinden emülsifiye sistemler, lipit nanopartiküller, lipozomlar, nanoyapılı lipit taşıyıcılar) yararlanılmıştır (Soudry-Kochavi ve ark., 2015; Suzuki ve ark., 2019; Xu ve ark., 2020; Celik-Tekeli ve ark., 2021). Nanopartiküller, genellikle doğal ve sentetik polimerlerden ya da inorganik bileşenlerden hazırlanan boyutları 1000 nm'nin altında olan kolloidal sistemler olarak tanımlanmaktadır. Nanopartiküllerin hazırlama yöntemi, hazırlama materyali ve yapısına göre nanoküreler veya nanokapsüller elde edilebilmektedir. Nanoküreler etkin maddenin polimerik küre içerisinde dağıldığı heterojen sistemlerdir. Nanokapsüllerde ise etkin madde polimerik zarfla çevrili bir çekirdek kısmında heterojen dağılmıştır. Çekirdek kısmı sulu veya lipofilik çözücülerden oluşmaktadır (Couvreur ve ark., 2002). Peptit/proteinleri parçalanmaya karşı koruyabilen ve onları gastrointestinal kanalda hedef bölgelere taşıyabilen nanopartiküler sistemler, daha etkili ve kontrollü ilaç salımını sağlayabilmektedirler (Ensign ve ark., 2012). Katı lipit nanopartiküller, biyouyumlu ve biyoparçanabilen katı lipitlerin yüzey etkin maddelerle stabilize edildiği nanoboyuttaki ilaç taşıyıcı sistemlerdir (Numanoğlu ve Tarımcı, 2006; Homan Gökçe ve Özer, 2014). Nanoyapılı lipit taşıyıcılar ise biyouyumlu ve biyoparçanabilen katı ve sıvı lipitlerin yüzey etkin maddelerle stabilize edildiği nanoboyuttaki ilaç taşıyıcı sistemlerdir (Chauhan ve ark., 2020). Lipozomlar, lipit çift tabaka ve sulu faz olmak üzere iki kısımdan oluşan küresel veziküllerdir. Lipozomların gastrointestinal kanal pH ve enzimlerden etkilenmeleri peptit ve proteinlerin oral yolla verilişini sınırlamaktadır. Lipozomların polimerlerle kaplanarak hem bu problemlerin aşılması hem de kontrollü salım sağlanması amaçlanmaktadır (Zırh-Gürsoy, 2002). Kendiliğinden nanoemülsifiye olabilen sistemler (SNEDDS); doğal veya sentetik yağlar, katı veya sıvı yüzey etkin maddeler, yardımcı yüzey etkin madde/ hidrofilik çözücü/ yardımcı çözücülerin izotropik/homojen karışımlarıdır. Bu sistemler, oral alımı takiben GİK'teki sulu ortamda damlacık büyüklüğü 300 nm'nin altında Y/S nanoemülsiyonları oluşturmaktadır. Bu formülasyon bağırsak lümenine salındığında, nanoboyutta emülsiyon oluşturmak üzere dağılır, bu sayede hidrofobik etkin madde bağırsakta çözelti içinde kalmaktadır. Böylece emilim hızını kısıtlayan dissolüsyon aşamasını geçerek bağırsaktan emilim sonrası daha tutarlı bir plazma konsantrasyon-zaman profiline ve artmış biyoyararlanıma ulaşılabilir (Pouton, 1997; Gursoy ve Benita, 2004; Arslan ve Tirnaksiz, 2013). Terapötik peptit/proteinlerin gastrik ortamdan korunması, yiyecek etkisine bağlı değişikliklerin azaltılması, hazırlanışlarının kolay ve ölçek büyütmeye elverişli olması, formülasyonun kapsül içerisinde verilebilmesi gibi üstünlükleri SNEDDS'leri oral yoldan terapötik peptit/proteinlerin uygulanmasında öne çıkarmaktadır (Date ve ark., 2010). Exendin-4'ün oral biyoyararlanımını artırmak üzere yukarıda açıklanan ilaç taşıyıcı sistemlerle yapılan çalışmalar Tablo 3'te gösterilmiştir.

Tablo 3. Exendin-4'ün oral biyoyararlanımını artırmak üzere ilaç taşıyıcı sistemlerle yapılan çalışmalar

İlaç Taşıyıcı Sistem	Sonuç	Kaynak
Lipit nanokapsül	Lipit nanokapsülün *in vivo* çalışmayla oral uygulanmasıyla farelerde GLP-1 sekresyonunun arttığı gösterilmiştir. Exendin-4'ün oral biyoyararlanımı %4 bulunmuştur.	(Xu ve ark., 2020)
Eudragit L:HPMC (0,75:1) a/a mikropartiküller içerisinde sodyum trimetafosfat kullanılarak çapraz bağlı albümin ve dekstran nanopartikülleri	Farmakokinetik verilere göre oral uygulanan Eudragit L:HPMC (0,75:1) a/a mikropartiküller içerisindeki exendin-4 yüklü dekstran-50/BSA nanopartiküllerinin Byetta™ subkütan enjeksiyonuna göre bağıl biyoyararlanımı %77 olarak bulunmuştur. Bu durumun nanopartikül matriks yüzeyindeki dekstranın enterositler tarafından lenfatik dolaşıma nanopartiküllerin geçmesini sağladığı için karaciğer ilk geçiş etkisini ortadan kaldırmasına bağlı olduğu belirtilmiştir.	(Soudry-Kochavi ve ark., 2015)
Eudragit L100-55 ile kaplanmış kitosan/poli-ɣ-glutamik asit (KS/ɣPGA) nanopartikülleri	Diyabetik sıçanlarda yapılan *in vivo* çalışmada oral yolla uygulanan exendin-4 içeren kitosan/ɣPGA nanopartiküllerinin serbest exendin-4'ün sc enjeksiyonuna göre bağıl biyoyararlanımı %14 bulunmuştur.	(Nguyen ve ark., 2011)
KS/ɣPGA nanopartikülleri	Exendin-4 içeren KS/ɣPGA nanopartikülleri ve insülin/exendin-4 içeren KS/ɣPGA nanopartiküllerinin diyabetik sıçanlarda insülin seviyesini anlamlı derecede artırırken glukagon seviyesini de anlamlı derecede azalttığı gösterilmiştir ($p<0.05$). İnsülin ve glukagon seviyelerindeki anlamlı değişikliğin exendin-4'ün endojen insülin sekresyonunu artırmasına bağlı olduğu belirtilmiştir. İnsülin ve exendin-4 içeren KS/ɣPGA nanopartiküllerinin ise insülin-KS/ɣPGA nanopartiküllerine ve exendin-4-KS/ɣPGA nanopartiküllerine göre hipoglisemik etkisi 2 kat fazla bulunmuştur.	(Chuang ve ark., 2013)
CSKSSDYQC (CSK) peptit spesifik ligandı kullanılarak goblet hücrelerine hedeflendirilebilen kitosan nanopartikülleri	Exendin-4 yüklü CSK-kitosan nanopartikülleri, exendin-4 çözeltisine göre nispeten yüksek hipoglisemik etki göstermiştir. CSK-kitosan nanopartiküllerinin bağıl biyoyararlanımı %6,56 olup, kitosan nanopartiküllerine göre 1,7 kat yüksek bulunduğu belirtilmiştir. CSK-kitosan nanopartiküllerinin goblet hücrelerine etkili bir şekilde hedeflendiği bu durumun oral peptit ve protein taşınmasında önemli olduğu belirtilmiştir.	(Li ve ark., 2015)
CSK peptidiyle modifiye edilmiş dekstran-PLGA nanopartikülü (DEX-PLGA NP)	Exendin-4 yüklü CSK-DEX-PLGA NP'leri exendin-4 yüklü DEX-PLGA NP'lerine göre exendin-4'ün biyoyararlanımını 1,6 kat artırmıştır. Exendin-4 yüklü CSK-DEX-PLGA NP'lerinin exendin-4'ün subkutan enjeksiyonuna göre bağıl biyoyararlanımı %9,2 bulunmuştur. Exendin-4 yüklü DEX-PLGA NP'leri ve exendin-4 yüklü CSK-DEX-PLGA NP'lerinin exendin-4 çözeltisine göre diyabetik sıçanlarda hipoglisemik etkisi anlamlı derecede artmıştır ($p<0,05$). Exendin-4 yüklü CSK-DEX-PLGA NP'lerinin exendin-4 yüklü DEX-PLGA NP'lerine göre hipoglisemik etkisi daha fazla bulunmuştur.	(Song ve ark., 2019)
Katı lipit nanopartiküller	INS-1 hücrelerinde sitotoksisitesi ve insülin sekresyonuna etkisi exendin-4 çözeltisiyle karşılaştırıldığında exendin-4 yüklü katı lipit nanopartiküllerinin sitotoksik etkilerinin gözlenmediği ve INS-1 hücrelerinde insülin sekresyonunu kolaylaştırdığı belirtilmiştir.	(Jun ve ark., 2015)
Kondroitin sülfat-glikolik asit kaplı lipozomlar	Sıçan modellerinde, oral yolla uygulanan exendin-4 yüklü kondroitin sülfat-glikolik asit kaplı lipozomlar (200μg/kg) subkutan exendin-4 uygulamasına (20 μg/kg) kıyasla %19,5 bağıl biyoyararlanım göstermiştir. Oral yolla uygulanan exendin-4 yüklü kondroitin sülfat-glikolik asit kaplı lipozomlar (300 μg/kg)), subkütan uygulanan exendin-4'e (20 μg/kg) göre diyabetik sıçanlarda kan glikoz seviyelerinde daha iyi düşüş sağlamıştır.	(Suzuki ve ark., 2019)
Nanoyapılı lipit taşıyıcılar	Exendin-4 yüklü nanoyapılı lipit taşıyıcıların endojen GLP-1 salımına etkisinin araştırıldığı *in vivo* çalışmada diyabetik olmayan farelerde kan glikoz seviyelerinde anlamlı bir düşüş gözlenmemiştir ($p<0.05$). Bu durumun kan glikoz seviyeleri normalden daha yüksek olduğunda GLP-1 agonisti olan exendin-4'ün hipoglisemik etkisini gösterebilmesinden kaynaklandığı düşünülmüştür.	(Shrestha ve ark., 2018)
Kendiliğinden emülsifiye olan nanoformülasyon (SNEDDS)	Diyabetik sıçanlarda exendin-4 içeren SNEDDS ve exendin-4/kimostatin içeren SNEDDS formülasyonlarının exendin-4'ün çözeltisine göre oral biyoyararlanımı sırasıyla 1,3 ve 1,6 kat artırdığı bulunmuştur. *In vivo* farmakodinamik çalışmada diyabetik sıçanlarda exendin-4/kimostatin SNEDDS ve Byetta® (sc) uygulanan grupta kan konsantrasyonuna paralel şekilde ilk 60. dakikada plazma insülin seviyelerinde belirgin bir artış gözlenmiştir. Diyabetik sıçanlara oral yolla uygulanan exendin-4 içeren SNEDDS ve exendin-4/kimostatin içeren SNEDDS formülasyonları, subkütan yolla uygulanan Byetta® 30. dakikada kan glikoz seviyelerinde sırasıyla %23, %25, %29 düşüş sağlamış ve elde edilen profiller Şekil 3'te gösterilmiştir.	(Celik-Tekeli ve ark., 2021)



Şekil 3. Diyabetik sıçanlara ait (a) exendin-4 plazma konsantrasyon-zaman profili, (b) plazma insülin seviyelerinde değişiklik yüzdesi-zaman profili, (c) kan glikoz seviyelerinde değişiklik yüzdesi-zaman profili (Celik-Tekeli ve ark., 2021 (Elsevier'den izinli)).

Exendin-4'ün Pulmoner Yolla Uygulanması

Akciğer, ilaç absorpsiyonu için geniş bir yüzey alanı oluşturmaktadır. Ayrıca alveolar epitel 0,1-0,5 µm kalınlığında olup hızlı ilaç emilimine izin vermektedir. Alveollere, kütle ortalama aerodinamik çapı (KOAÇ) 5 µm'den az olan bir preparat aerosol olarak verilerek ilacın emilimi için etkili bir şekilde hedeflendirilmiş tedavi sağlanabilmektedir. Ayrıca, pulmoner yolla verilişte gastrointestinal sistemin ilk geçiş etkisinden de kaçınılmaktadır. Metabolik enzimler akciğerlerde bulunabilmesine rağmen, metabolik aktiviteler ve yollar, gastrointestinal sistemde gözlemlenenlerden farklı olabilir, bu da birçok peptit ve proteinin pulmoner uygulamasını umut verici hale getirmektedir (Agu ve ark., 2001). Polimerik mikro/nanopartiküller ve nanojeller kullanılarak exendin-4'ün pulmoner yolla verilişiyle ilgili çalışmalar yapılmıştır. Nanojeller, şişme özelliğine ve ağsı yapıya sahip spesifik polianyonlarla katyonik polimerlerin bağlanması ya da polimerlerin çapraz bağlanmasıyla hazırlanabilen polimerik nanotaşıyıcılardır (Çelebi, 2014). Pulmoner yolla verilmek üzere hazırlanan exendin-4 içeren ilaç taşıyıcı sistemler Tablo 4'te gösterilmiştir.

Tablo 4. Pulmoner yolla verilmek üzere hazırlanan exendin-4 içeren ilaç taşıyıcı sistemler

İlaç Taşıyıcı Sistem	Sonuç	Kaynak
Deoksikolikasit-glikol-Kitosan kaplı nanojeller	Diyabetik farelere inhalasyon yoluyla verilen palmitik asit exendin-4 konjugasyonu (Pal-Ex-4) deoksikolikasit-glikol-kitosan nanojellerinin palmitil açilasyonu yapılmayan deoksikolikasit-glikol kitosan nanojellere göre daha iyi hipoglisemik etki gösterdiği belirtilmiştir.	(Lee ve ark., 2012)
Pal-Ex4 içeren albumin kaplı PLGA mikropartikülleri	Palmitil açilasyonu exendin-4 konjugatının plazmada dolaşımını artırmıştır. Ortalama aerodinamik çap 3,2±0,3 µm bulunmuştur. Mikropartiküllerin albüminle kaplanması aerolize olan miktarı 1,6 kat artırmıştır. Palmitil açilasyonu yapılmış exendin-4'ün albüminle kaplanmış PLGA mikropartikülleri diyabetik farelerde 5 gün boyunca hipoglisemik etki sağlamıştır.	(Kim ve ark., 2011)
Pal-Ex-4 kitosan-PLGA nanopartikülleri	Diyabetik farelere inhalasyon yoluyla verilen Pal-Ex-4 kitosan-PLGA nanopartiküllerinin hipoglisemik etkisi kitosan exendin-4 PLGA nanopartiküllerinden 3,1 kat daha iyi bulunmuştur.	(Lee ve ark., 2013)

Exendin-4'ün Diğer Uygulama Yolları

Parenteral, oral, pulmoner yolla dışında intranazal ve transdermal yol peptitlerin verilişi için birçok avantaj sunmaktadır. Nazal uygulama, burun boşluğunun nispeten geniş yüzey alanı sayesinde etkin maddenin emilimini kolaylaştırması, burun dokusunun oldukça geçirgen ve vasküler yapısı, gastrointestinal ortamın proteolitik etkisinden ve hepatik ilk geçiş metabolizmasından kaçınma dahil olmak üzere peptit/proteinlerin sistemik olarak verilebilmesi için çeşitli avantajlar sunmaktadır (Al Bakri ve ark., 2018). Ayrıca nörolojik bozukluklarda nazal uygulama beyne hedeflemede önemli avantajlar sağlamaktadır. Bir çalışmada C57BL/6J farelerine orta serebral arter oklüzyonu ameliyatından önce 7 gün boyunca exendin-4 intranazal yolla uygulanmıştır. İntranazal olarak uygulanan exendin-4, intraperitoneal olarak uygulanan exendin-4'le karşılaştırıldığında beyinde daha yüksek konsantrasyonlarda exendin-4 bulunduğu saptanmıştır. Enfarktüslü lezyonların hacmi iskemiden 24 saat sonra analiz edildiğinde exendin-4'ün intranazal uygulanması enfarktüs hacmini azaltarak farelerde önemli derecede nöroproteksiyon sergilemiştir. Exendin-4'ün pro ve anti-apoptotik proteinler arasındaki dengeyi yeniden kurarak kaspaz-3 ekspresyonunu azalttığı ve böylece nöroproteksiyon sağladığı belirtilmiştir (Zhang ve ark., 2016). Peptitlerin nazal yolla verilişi için ısıya duyarlı hidrojeller üzerinde çalışılmaktadır. Isıya duyarlı hidrojeller vücut sıcaklığında sol-jel geçişi meydana gelmesiyle ilaç salımı için bir depo görevi görmektedirler. Hazırlama aşamasında organik çözücülerin kullanılmaması ve üretim sürecinde yüksek sıcaklık gerekmemesi sayesinde peptitler gibi hassas terapötikler denatürasyona karşı korunmaktadır. Isıya duyarlı hidrojellerin; kontrollü salım sağlanması için depo sistem olması uygulama sıklığını en aza indirmekte ve hasta uyuncunu artırmaktadır (Bolhassani, 2019; Eissa ve ark., 2021).

Transdermal uygulama, peptitin derinin geniş yüzey alanı sayesinde epidermal tabakadan serbest difüzyon veya başka yollarla geçmesine izin vererek kontrollü bir şekilde sistemik kan dolaşıma girmeye devam etmesi, gastrointestinal ortamın proteolitik etkisinden ve hepatik ilk geçiş metabolizmasından kaçınılması gibi birçok avantajlar sağlamaktadır. Ayrıca subkütan enjeksiyonla karşılaştırıldığında, transdermal uygulama daha az travma ve daha düşük enfeksiyon riski, daha düşük maliyet ve yüksek hasta uyuncu sağlamaktadır. Diğer yandan derinin stratum korneum tabakası peptitlerin terapötik etki sağlayacak miktarda geçişini engelleyebilmektedir. Bu nedenle deriden geçişin artırılması için çeşitli stratejiler geliştirilmiştir (Long ve ark., 2020). Mikroiğneler, özellikle makromoleküllerin deriden geçişini artırmada önerilmektedir (Prausnitz ve ark., 2004). Transdermal ve intranazal yolla verilmek üzere hazırlanan exendin-4 içeren ilaç taşıyıcı sistemler Tablo 5'te gösterilmiştir.

Tablo 5. Transdermal ve intranazal yolla verilmek üzere hazırlanan exendin-4 içeren ilaç taşıyıcı sistemler

İlaç Taşıyıcı Sistem	Sonuç	Kaynak
Karboksimetil selüloz bazlı çözünebilen mikroiğneler	Çözünebilen mikroiğnelerin imalatında yer alan parametreleri optimize etmenin, exendin-4'ün aktivitesini %98,3 ± 1,5'e kadar koruduğu gösterilmiştir. Ayrıca exendin-4 yüklü çözünebilen mikroiğnelerin diyabetik farelerde kan glikoz seviyesini subkütan enjeksiyona benzer şekilde düşürdüğü belirtilmiştir.	**(Fakhraei Lahiji ve ark., 2018)**
Hyaluronik asit bazlı çözünebilen mikroiğneler	Exendin-4 içeren çözünebilen mikroiğnelerin hazırlandığı çalışmada yüklü mikroiğne dizilerinin uygulanmasından sonra kan glikoz seviyesinin düştüğü, insülin sekresyonunun arttığı belirtilmiştir. Bu etkilerin exendin-4'ün subkütan enjeksiyonundan sonraki etkilerle karşılaştırılabilir bulunduğu belirtilmiştir. Exendin-4 yüklü mikroiğne dizilerinin Tip 2 diyabetik sıçanlara uygulanmasından sonra exendin-4'ün subkütan enjeksiyonuna benzer plazma konsantrasyon profilleri gösterdiği belirtilmiştir.	**(Liu ve ark., 2016)**
Aljinat bazlı çözünmeyen mikroiğneler içeren deri örtüsü	Exendin-4 içeren pH'ya duyarlı olan çözünebilen mineralize kalsiyum fosfat partikülleri (m-Ex4) hazırlanmıştır. Hem mineralize glikoz oksidaz (m-GOx) hem de m-Ex4, aljinat bazlı çözünmeyen mikroiğneler içeren deri örtüsüne entegre edilmiştir. Hiperglisemik koşullarda, m-GOx'un, glikoz sinyallerini H+ sinyallerine dönüştürerek ve m-Ex4 partiküllerinin ayrışmasını tetikleyerek exendin-4'ü serbest bıraktığı belirtilmiştir. Bu sayede kan glikoz seviyesinin 5 gün boyunca normal düzeyde kaldığını belirtmişlerdir.	**(Chen ve ark., 2017)**
Magnezyum klorür içeren kitosan gliserofosfat bazlı hidrojel	Magnezyum klorür içeren kitosan gliserofosfat bazlı hidrojeli, sıçanlara nazal yolla uygulandığında exendin-4 çözeltisine göre bağıl biyoyararlanımın arttığı belirtilmiştir. Yüksek yağlı diyetle beslenen sıçanlarda hidrojel on gün içinde, gıda alımını önemli ölçüde azaltırken kilo kaybında da anlamlı bir değişiklik sağlamıştır ($p< 0.05$).	**(Li ve ark., 2018)**
Tiyollenmiş poliakrilik asit mikropartikülleri	Domuz nazal mukozası üzerinde yapılan *ex vivo* çalışmada, permeabilite katsayısı exendin-4 içeren tiyollenmiş mikropartiküllerde exendin-4 çözeltisine kıyasla 4,7 kat daha fazla bulunmuştur.	**(Millotti ve ark., 2014)**

SONUÇ

Tip 2 diyabet tedavisinde inkretin bazlı terapiler son yıllarda önem kazanmıştır. En yaygın kullanılan ilaçlardan biri olan exendin-4'ün parenteral yolla uygulanan Byetta® ve uzun etkili Bydureon™ ticari ürünü bulunmaktadır. Ayrıca exendin-4'ün parenteral yolla verilmesinin bazı sakıncalarından dolayı alternatif yollarla ilgili çalışmalar yapılmaktadır. Yukarıda açıklanan çalışmalarda görüldüğü gibi, ilaç taşıyıcı sistemler kullanılarak farklı uygulama yollarıyla exendin-4'ün antidiyabetik etkinliği değerlendirilmiştir. Yapılan çalışmalar değerlendirildiğinde; bulunan sonuçların exendin-4'ün parenteral uygulamaya alternatif olarak oral, pulmoner, transdermal vb. gibi farklı veriliş yollarıyla exendin-4'ün ticari ürünlerinin geliştirilmesinde ve yeni inkretin bazlı peptit-proteinlerle yapılacak çalışmalar için de umut vadedici olacağı düşünülmektedir.

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