Journal of Advanced Biomedical and Pharmaceutical Sciences

Journal Homepage: http://jabps.journals.ekb.eg

Formulation and clinical evaluation of niosomal methylene blue for successful treatment of acne

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Received: March 13, 2020; revised: April 7, 2020; accepted: April 7, 2020

Abstract

Photodynamic therapy (PDT) can be defined as the administration of a nontoxic drug or dye known as Photosensitizer (PS) either systemically, locally, or topically to a patient bearing a lesion. Methylene blue considered as a photosensitizer for photodynamic therapy**.** Current topical and oral therapies for acne vulgaris have limited efficacy, especially in moderate to severe cases,

In view of this, the aim of our study was to use methylene blue in the form of niosomal hydrogel for photodynamic treatment of acne. To reach this objective we studied the following aspects; formulation of MB blue in different niosomal preparation, characterization of methylene blue niosomes, Span 60 transition temperatures, In vitro release study, kinetic analysis of the release data, factors affecting the encapsulation of methylene blue in niosomes via effect of: amount of drug, cholesterol: span 60 ratios, and stabilizers.

The results revealed that, niosomes were successfully produced by reversed phase evaporation technique using different ratio of cholesterol: span 60.The most favorable amount of MB could be used in niosomal preparation was 1000µg. The best fit kinetic favored Higuchi diffusion mechanism. The incorporation of MB niosomes in HPMC 3% gel resulted in feasible release rate of MB. The data obtained served as the basis to reach a secondary objective which is clinical evaluation of selected niosomal gel of methylene blue for photodynamic treatment of acne which showed higher significant improvement in inflammation when compared with IPL treatment.

Key words
Niosome, methylene blue, photodynamic therapy, acne

1. Introduction

PDT is a promising manner for the management of various tumors and nonmalignant diseases, based on the combination of a photosensitizer that is selectively localized in the target tissue and illumination of the lesion with visible light, resulting in photo damage and subsequent cell death. Numerous worldwide clinical trials have shown that PDT represents an effective and safe modality for various malignant conditions [1-4]. PDT is an effective and safe for various malignant conditions [5-8].

MB is histological dye used for many years [9, 10]. It belongs to the phenothiazinium class of compounds. The specific color of MB is due to the strong absorption band in the 550-700 nm regions with maximum molar absorptivity of 85,000 M−1 cm−1 at 664nm [11]. Methylene blue is a molecule having vital roles in microbiology and pharmacology; moreover it is used to stain living organisms considered as a Photosensitizer for photodynamic therapy [12-16].

Niosomes (the nonionic surfactant vesicles) can improve the solubility as well as the stability of pharmaceutical molecules since niosomes can act as drug reservoirs, can be a carrier for hydrophilic and hydrophobic drugs and has the adjusting effect on drug release rate [17-20]. Several factors can effect on niosomes construction such as the method of preparation, types and amounts of surfactants, entrapment of drug, temperature of lipids hydration, and the factor of packing.

Particular efforts have been targeted to use niosomes as an effective transdermal and dermal drug delivery systems [21, 24]. In particular, topical application of niosomes has an increasing effect on the residence time of drugs in the stratum corneum and epidermis. Moreover, non-ionic surfactants normally show favorable dermatological properties [16].

Skin is a vital organ offering an appropriate site for administration of many drugs. However, the most disadvantages of transdermal drug delivery are the low rate of penetration of drugs via the skin. Drugs encapsulated in nanoparticulate vesicles permits the transports of drugs into the skin [25-26]. Therefore, niosomes are interesting and open a key window to explore the possibility of using for the topical delivery of active compounds as carriers. In the present study, we prepared niosomes to encapsulate MB.

Acne vulgaris is defined as chronic inflammatory disease of the pilosebaceous unit resulting from various interacting pathophysiologic factors [27-31]. Nowadays topical and oral

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treatment for acne vulgaris have minor efficacy particularly, in moderate to severe cases [29]. The resistances of antibiotic as well as the challenges of isotretinoin treatment have led to fulfillment of PDT in the treatment of acne vulgaris [28]. Photodynamic therapy may alter many of the acne vulgaris pattern, as it's defined in the treatment of moderate to severe inflammatory acne vulgaris.

In this study we use methylene blue in the form of niosomal hydrogel for successful photodynamic treatment of acne as it is need deep penetration through the skin.

2. Experimental

2.1. Materials

Standard semi-permeable cellulose membrane (12000-14000 MWCO) (Sigma Chem. Co., USA). Hydroxyethyl cellulose (HEC) (EL-Naser Pharm. Chem. Co., Cairo, Egypt). Hydroxy propyl methylcellulose viscosity 15000 mpa.s (HPMC) (Alpha chem. Mumbai, India). Methanol, potassium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride and potassium chloride (United Co., Chem. and Med. Prep., Egypt).Diethyl ether and Span 60 (Adwic, El-Naser chemical co., Egypt).Methylene blue (MB)(Sigma-Aldrich, St Louis, MO, USA).Cholesterol, Dicetyl phosphate (DCP) and Pluronic F-127 (Sigma Chem. Co., USA).

2.2. Methodology

2.2.1. Preparation of MB-loaded niosomes

Niosomal vesicles were prepared using reversed phase evaporation method [9] where different molar ratios of cholesterol to Span 1:1, 1:2, 1:3 and 2:1 were examined; the total concentration of cholesterol and Span 60 was kept at 240 µM. Briefly an appropriate amounts of cholesterol and span 60 were dissolved in 15ml of diethyl ether in 250 ml, then 5ml of phosphate buffer saline pH (7.4) containing MB added to organic phase to form organic-to-aqueous-phase ratio 3:1.The organic solvents were discarded under vacuum in a rotary evaporator model R200 (BÜCHI Labortechnik AG, Switzerland) at 50 rpm for 20 minutes, Then the resulting solution kept rotating for 30 minutes in rotary evaporator at 90 rpm under normal pressure, the dispersion of niosomes was set in the refrigerator at 4 °C overnight.

2.2.2. Separation of free methylene blue from niosomal suspension

Non-entrapped drug was separated by centrifugation at 14,000 rpm for 30 min at 4 °C via cooling ultracentrifuge (Cooling Ultracentrifuge. Model 8880, Centurion Scientific Ltd., W. Sussex, UK). The supernatant was then discarded and the residue was undergoing washing with phosphate buffer saline (PBS) of pH 7.4 for three times. Re centrifugation was carried out after each step of washing.

2.2.3. Determination of entrapment efficiency (EE)

The concentrations of entrapped MB were determined as follows: the collected residue after centrifuge was lysed in methanol and sonicated for five minutes to get clear solution. The concentration of methylene blue was determined spectrophotometrically at λ max 664 nm [32, 33].The percentage of entrapment efficiency of MB was determined according to the following equation:

$$\% \text{ EE} = \frac{\text{Amount loaded of MB in niosomes}}{\text{Total amount of added MB}} \text{ x } 100$$

3. Characterization of methyleneblue niosomes

3.1. Measurements of zeta-potential and particle size

Dynamic light scattering measurements were carried out using a Malvern Zeta Sizer (Nano-ZS, Malvern Instruments, Worcestershire, UK). The instrument was equipped with a 4mW helium/neon laser (λ = 633nm). Zeta potential and size of particles were measured at 25 °C.

3.2. Photo microscopic Studies

Samples of MB niosomes (freshly prepared) were investigated using TEM and microscopically at magnification of 40× using light microscope (Olympus Cx41RF, Tokyo. Japan).

3.3. In vitro release of methylene blue from noisome suspension

This study was carried out for each niosomal formulation using niosomal suspension carrying fixed amount of MB (1 mg) as follow: One gram of suspension of noisome was put on a circular area (6 cm^2 diameter) of moistened cellophane membrane with the receptor phase, and firmly stretched over one end of glass tube. The tube was then soaked in a 100 ml beaker containing 50 ml of the release media (phosphate buffer pH 6.8) and immersed in thermostatically controlled water bath fixed at 50 rpm at 35±2 °C. Five (ml) of sample was withdrawn at definite time intervals for 2 hours and then replaced by same volume of the release medium [34].

3.4. Determination of span60 transition temperature

DSC measurements were investigated using differential scanning calorimeter (DSC-50; Shimadzu, Japan) calibrated with indium. DSC measurements were investigated for niosomal preparations and the excipients (as pure chemicals) where 3-5 mg sample was sealed in standard aluminum pan and heated up to 100 °C. The thermograms were obtained at constant increasing rate of 5 °C/min in a nitrogen flow rate 20 ml/min. followed by determination of the maximum endothermic peak of Span 60.

3.5. Factors affecting the encapsulation of methylene blue in niosomes.

With regard to the amount of drug, the increasing effect of MB content on the EE in the range of 500 – 2000 µg in the noisome prepared at different ratios of cholesterol: span 60 was

determined. To elucidate the influence of stabilizers, the effect of DCP a negatively charged molecule on the particle size and entrapment efficiency was evaluated. On the other hand, since cholesterol can increase the rigidity of the bilayer of niosomes [35].The effects of increasing cholesterol content on drug entrapment in niosomes were investigated where different molar ratios of cholesterol to span 60 (1:1, 1:2, 1:3, 2:1) were prepared. However, for each niosomal preparation we determined the encapsulation efficiency, particle size and zeta potential.

3.6. Dispersion of noisomal methylene blue in formulations of gels

To facilitate the application of niosomal formulations to patients as well as to control release of drug, the selected dispersion of noisome (N2), was chosen to be formulated in three gel matrices. The study was based on the in vitro drug release of MB from different types of gel formulations as well as kinetic analysis of release data and compared between gel formulations. The types of gel formulations studied were HPMC (3 %), HEC (3 %) and Pluronic F127.

In case of pluronic F-127 hydrogel formulation, the weighed amount of this polymer was added slowly with stirring to cold phosphate buffer containing MB previously dissolved in it and set over night in the refrigerator to ensure complete dissolution of the polymer. After that the solution were left outside the refrigerator at room temperature to obtain hydrogel [36].

For HEC and HPMC hydrogel, the amount of the polymer was dispersed slowly in water containing MB previously dissolved in it. The dispersion was slowly stirred by magnetic stirrer. Then the dispersion was left at room temperature overnight, for complete swelling [35].

3.7. In vitro release of MB from gel formulations

This study was done using 1g of gel of PF127 (20 %), HPMC (3 %), HEC (3 %) formulation contain 100 µg of MB under investigation using the same procedure mentioned previously in vitro release of methylene blue from noisome suspension. The amount of MB released at time intervals was determined spectrophotometrically at λ max 664 nm.

3.8. Physical stability

The selected MB -loaded noisome was investigated for stability after storage. Stability test examined via visual observation, drug content, particle size, and zeta potential. Hence, the selected MB loaded niosome in colored glass vials was set in refrigerator at 5 ± 3 °C for three months. Samples were investigated at the end of first and third months.

3.9. Data analysis

Analysis of data were done with SPSS 21 software using one way analysis of variance (ANOVA), followed by LSD Post Hoc Test.

3.10. Patients and Methods

Forty five patients presented by inflammatory facial acne vulgaris were included in the study. The selected candidates for this study were patient's attending the outpatient clinic of Dermatology, Venereology and Andrology, Al-Azhar (Assiut) university hospital.

The group study included forty five patients (9 males and 36 females), aged 17- 28 years, the duration of disease ranged from 7 months to 8 years. Patients were classified according to Evaluator Global Severity Score (EGSS) of acne vulgaris (Eichenfield *et al*.,) [37], into mild degree (n=5), moderate degree (n=27) and severe degree (n=13).

Table 1: Evaluator Global Severity Score (EGSS)

Score	Grade	Description
0	Clear	Normal, clear skin with no evidence of acne vulgaris.
1	Almost Clear	Rare comedones present, with rare papules (papules must be resolving and may be hyperpigmented, though not pink-red).
2	Mild	Some comedones are present, with few inflammatory lesions (papules/pustules only; no nodules).
3	Moderate	Comedones predominate, with multiple inflammatory lesions evident; several to many comedones and papules/pustules; there may or may not be one small nodule.
4	Severe	Inflammatory lesions are more apparent, many comedones and papules/pustules, there may or may not be a few nodules.
5	Very Severe	Highly inflammatory lesions predominate, variable number of comedones, many papules/pustules, and many nodules.

3.11. Exclusion criteria

This is including the following exclusion criteria: Oral isotretinoin for the past 6 months, photosensitive dermatoses, pregnant or lactating women, Hypertrophic scars or keloids and history of polycystic ovary.

3.12. Each patient was subjected to the following

3.12.1. Complete history taking included

Personal history include: name, age and sex, present history of acne as regards the age at onset, duration, course of the disease and aggravating factors, family history of acne vulgaris. Also, past history of any associated disease either involving the skin e.g. hirsutism and androgen etic alopecia or non-skin disease e.g. endocrinal disease and polycystic ovary. Moreover the previous forms of therapy either systemic or topical and degree of response to such treatment.

3.12.2. Examination included

This examination included: general examination for endocrinal disturbance (androgenic alopecia or hirsutism), local examination such as: the type of lesions either inflammatory (papules, pustules, nodules and cysts) or non-inflammatory (comodones) and their count. However, the global severity of acne was assessed by the investigator using a six-point rating scale; Evaluator Global Severity Score (EGSS) **(Table 2)**. Patients included in the study had a Global Severity Score 2, 3 and 4 before treatment

3.12.3. Treatment protocol

Explanation of the procedure to every patient included in this study with all possible cosmetic procedure results and occurrence of complications, whether transient or persistent, and consent was obtained. Then photographing the patients was examined using camera Olympus c- 420 digital SLR camera 10MP before and 2 weeks after each session.

3.12.4. Photodynamic therapy

Both the patient and physician wear specific (goggles) to protect against harmful effects of laser on eyes then a topical niosomal gel of MB was applied on the face 60 min before the treatment. KY gel was applied before the laser treatment. IPL laser was applied to left side of the face. Sun screen of (SPF 50 %) was applied after each session. Each patient was treated with three sessions at one week interval and was clinically evaluated at baseline, before each treatment and two weeks after the third treatment session. (Source of light: IPL (DEKA– ITALY.)

In case of intense pulsed light (IPL side): using equipment: IPL - DEKA -ITALY. K Y gel was applied over right side immediately before IPL, while IPL was applied to right side of the face. Also here sun screen of (SPF 50 %) was applied after each session. Right side of the face was treated with IPL alone while left side was treated with MB mediated photodynamic therapy.

The treatment fluencies were 13-16 J/ (cm^2) according to skin type of patient and pulse width was 20ms and 8 cm^2 spot sizes. The 550 hand piece was used throughout the study and patients received 2 passes at each treatment session. Evaluations included formal counts of inflammatory lesions. Clinical improvement was assessed by a global grading scale [38]. Physicians investigated the side effects such as erythema, edema, hyper or hypopigmentation.

3.12.5. Follow-up included

This was assessed via comparing the photographs before and after therapy, appearance of new lesions and possible side effects. The patients will following up every 4 week for 4 months.

3.12.6. Statistical analysis

The results of the current study were checked, coded and analyzed using SPSS version 21 software. Results were assessed as simple percentage associated by qualitative description of comments. t test was used to determine the significance of differences between the data of the studied groups.

Table 2: Global grading scale

Number	Degree	Percent of improvement
1	Clear	100%
2	Almost clear (Excellent)	(75% - <100%)
3	Marked improvement (Very good)	(50 - <75%)
4	Moderate improvement (Good)	(25 - <50%)
5	Slight improvement (Poor)	(1 - < 25%)
6	No change	0%

4. Results and discussion

4.1. Characterization of methylene blue niosomes

The compositions and characteristics of the niosomal formulations are shown in **(Table 3)**. Niosomes were successfully produced by reversed phase evaporation method. Microscopical analysis of freshly prepared niosomes showed rounded vesicles **(Figure 1)**. It was found that, the mean particle size of the prepared niosomes ranged from 215.3±7.9 nm to 1273± 26.8nm , entrapment efficiency from 39.51±3.4% to 71.14±4.6% and zeta potential ranged from -33.2±3.1 to -80.9±5.3.

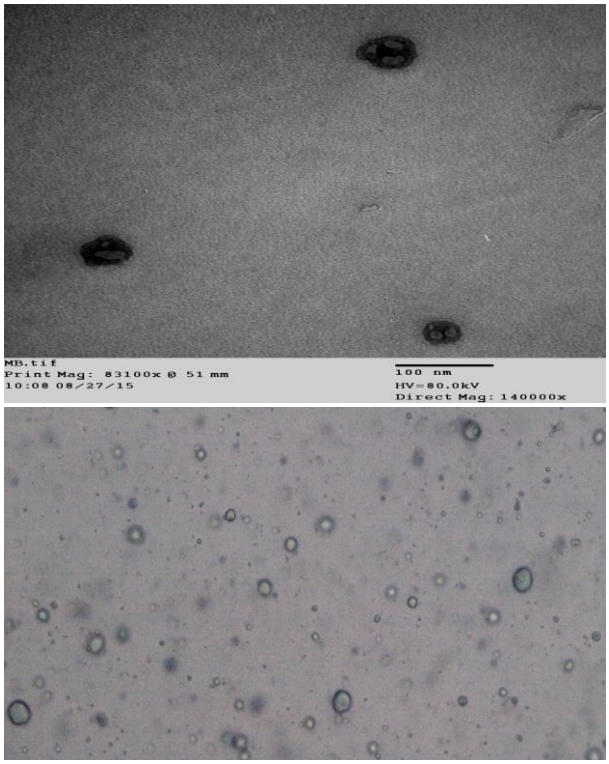

Figure 1: Photograph of freshly prepared niosomes of MB prepared by reverse phase evaporation technique

Table 3: Compositions and characteristics of the niosomal formulations

Niosome formulation	Cholesterol:span 60(molar ratio)	Dicetyl phosphate (µm)	Entrapment efficiency EE (%)	Niosome size(nm)	Zeta potential	PDI
N1	1:1	-	56.23±3.2	254.4±6.8	-39.5±1.8	0.335±0.006
N2	1:2	-	67.67±4.1	328.3±8.2	-45.6±2.3	0.497±0.001
N3	1:3	-	69.09±2.6	529.7±10.6	-49.3±2.1	0.611±0.009
N4	2:1	-	39.51±3.4	215.3±7.9	-33.2±3.1	0.240±0.002
N5	1:1	10	61.06± 4	615.9±12.5	-54.7±1.4	0.569±0.001
N6	1:2	10	70.51±3.8	1238±30.6	-66.3±4.2	0.468±0.005
N7	1:3	10	71.14±4.6	1273±26.8	-80.9±5.3	0.326±0.003
N8	2:1	10	46.81±1.5	543.3±14.1	-45.4±3.5	0.534±0.007

It has been reported that the effect of drug on the mean size and size distribution of vesicle was the result of an interaction of the compound with the bilayer structure [39, 40]. The increase in median diameter due to expand of vesicles as a result of entrapment of more methylene blue molecules either within the bilayers or inside the core of the niosomes.

In this study we use span 60 for the following reasons: 1) Span 60 has high phase transition temperature and so high entrapment efficiency. 2) Span 60 has long saturated alkyl chain (C16) results in high entrapment efficiency since as length of surfactant increases, entrapment efficiency also increases. 3) Also, the longer alkyl chain influences the HLB value of the surfactant mixture which in turn directly influences the drug entrapment efficiency. The lower the HLB of the surfactant the higher will be the drug entrapment efficiency and stability 60 [41]. Delete or add in introduction.

4.2. Span60 transition temperature

DSC analysis revealed that, the cholesterol content in the formulation changed the transition peak of Span 60 in the niosomal formulations. Generally, when the molar ratio of cholesterol increased, the endothermic peak temperatures decreased **(Table 3 and Figures 2and 3)**.

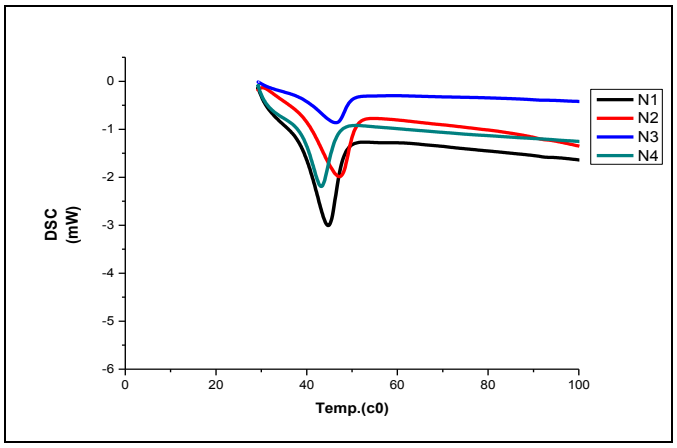

Figure 2: DSC thermograms of niosomes (N1-N4.)

Note: no extra peaks in the formulations

Cholesterol interacts with the other components in the niosomal formulations, which in turn alter the rigidity of bilayer membrane [42].This is a desirable feature since the leakage of content from the niosomes can be decreased [43].

The absence of extra endothermic peaks indicating a complete miscibility amongst the compositions of niosomes **(Figures 2&3)**. Finely, a higher drug EE was obtained when a higher transition temperature of Span 60 was found **(Table 3)**. Uchegbu and Vyas [44], reported that the higher transition temperatures of the surfactant, the less leaky vesicles were observed.

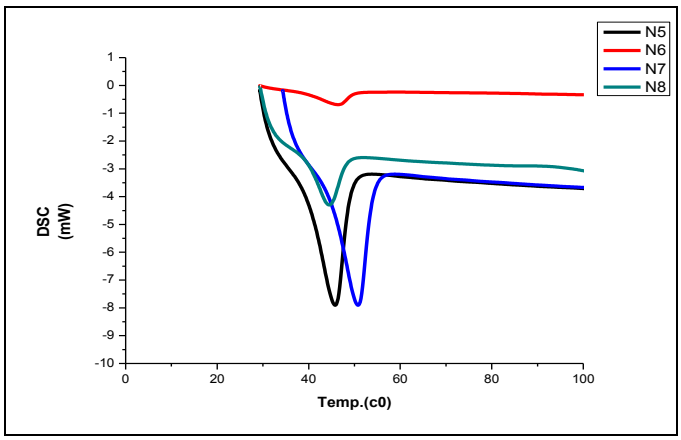

Figure 3: DSC thermograms of niosomes stabilized using DCP (N5-N8)

4.3. In vitro release study

The release of MB from niosomal vesicles of different span 60: cholesterol molar ratios are shown in **(Figure 4)**. It is clear that, there is no significant difference of in vitro release data between niosomal formulations containing DCP and that without DCP, $p \geq 0.05$ (N5 vs. N1), (N7 vs. N3) and (N8 vs. N4) except formulation N2 which shows a significant higher release of MB than other formulations $p \leq 0.05$. However the molar ratio of cholesterol: span60 2:1 represented by formulations N4&N8 gave the lowest entrapment, smallest particle size and slowest release. These results were in accordance with Mavaddati et al. [44] who reported that, cholesterol reduces the leakage or permeability of encapsulating drug via decreasing the niosomal membrane fluidity.

5. Factors affecting the encapsulation of MB in niosomes

5.1. Amount of drug

The entrapment efficiency of MB increases in Span 60 niosomes on increasing the drug concentration from 500 to 1000

µg **(Figure 5)**. The increased entrapment efficiency of MB with higher amount of drug used in the formulation may be due to the saturation of the hydration media with MB that forces the drug to be encapsulated into niosomes [45]. However, further increase in drug concentration from 1000 to 2000µg gave rise to a significant decrease in the entrapment efficiency ($P < 0.05$). This may be due to the fact that, the saturation of the bilayers of Span niosomes might be reached at 1000 µg of drug incorporation or niosomes reach its higher capacity of encapsulation. From the above results, it was concluded that increasing amount of drug has effect on entrapment efficiency and 1000µg showed the most favorable amount of MB could be used in niosomal preparation [46].

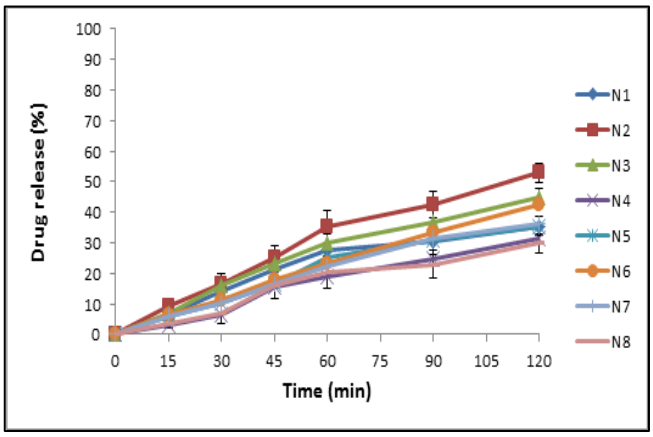

Figure 4: In vitro release profiles of MB from MB Niosomes (N1-N8)

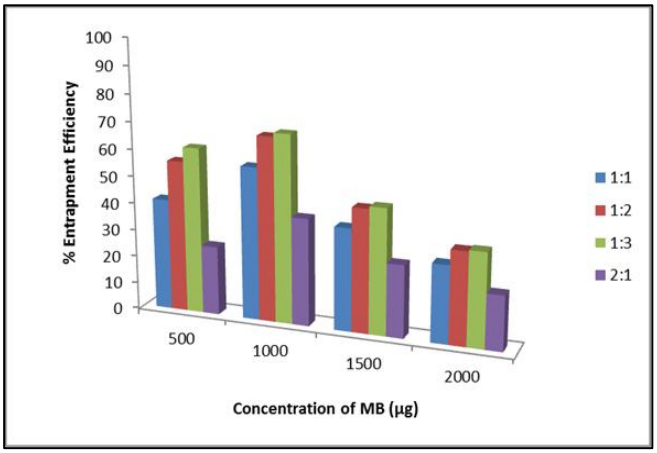

Figure 5: Effect of amount of drug on the entrapment efficiency of MB from niosomes prepared using different cholesterol : span 60 molar ratio (1:1,1:2,1:3,2:1).

5.2. Cholesterol: span 60 ratios

The entrapment efficiency is the most vital parameter from pharmaceutical viewpoint in niosomal formulations. Since, the times as well as the effort that involved in separation or removal of unentrapped material will greater decrease with the higher percentage of entrapments. **(Table 4)** illustrates series molar ratio formulations of cholesterol to span 60 at fixed amount of MB in order to elucidate the effect of cholesterol on the amount of drug entrapment in niosomes and proved that, the EE decreases significantly on increasing the cholesterol content in the formulation. The entrapment efficiencies (% EE) of MB were varied between 39.51±3.4% - 69.09±2.6% for niosomes (N1-N4) and 46.81±1.5% -71.14±4.6 for niosomes (N5-N8) containing DCP.

One-way ANOVA revealed that when, the molar ratio of cholesterol to Span 60 was <1.0 (N2, N3, N6, N7) the %EE of methylene blue was not statistically different (N2 vs. N3, p = 0.628), (N6 vs. N7, p = 0.829), (N2 vs. N6, p = 0.338), (N2 vs. N7, p = 0.245), (N3 vs. N6, p = 0.628) and (N3vs. N7, p = 0.486). However, when the ratio ≥ 1the %EE (N1, N4, N5, N8) were significantly smaller than other formulations ($p \leq 0.05$). In general, the vesicles became smaller as cholesterol content increased [47].

There are many reasons for the lower EE with higher cholesterol molar ratio. Since at higher cholesterol ratio, there are competition between cholesterol and the drug in the bilayer membrane of the niosomes for packing space, also, the bilayer hydrophobicity and stability increased and permeability decreased which may give rise to efficient trapping the hydrophobic drug into bilayers as vesicles formed. In addition, an increase in cholesterol molar ratio above a particular concentration could cause disruption of the structure of the vesicles formed and consequently, fewer drugs would be entrapped in the niosomes [48, 49].

Table 4: Effect of different molar ratio of cholesterol: span60 on the transition temperature of span 60

Niosome formulation	Cholesterol: span 60(molar ratio)	Dicetyl phosphate (µm)	Entrapment efficiency EE (%)	Span 60 (peak c^0)
N1	1:1	-	56.23±3.2	44.61
N2	1:2	-	67.67±4.1	48.01
N3	1:3	-	69.09±2.6	48.23
N4	2:1	-	39.51±3.4	43.59
N5	1:1	10	61.06± 4	45.26
N6	1:2	10	70.51±3.8	48.84
N7	1:3	10	71.14±4.6	50.45
N8	2:1	10	46.81±1.5	44.20

5.3. Stabilizers

The median diameter of the niosomes is greatly affected by the stabilizers in the preparations. DCP, negatively charged molecule was used in this study. The particle size of noisome containing DCP at different molar ratios of cholesterol to Span 60 revealed a higher significant increase in comparison with its related formulations without DCP $p \leq 0.001$(N5 vs. N1& N6 vs. N2&N7 vs. N3&N8 vs. N4). It has been reported that, the hydrophilicity of the bilayers of vesicles increased upon amalgamation of charged molecules in the bilayer which in turn enhanced water uptake into the bilayers of the vesicles. Furthermore, charge repulsion caused by charged species of DCP would separate the adjacent bilayers in the vesicles [50] , and as a result giving rise to larger vesicles and

consequently more drugs was entrapped as EE is directly related to the size of the niosomes [51].

The higher EE of MB in the prepared niosomes observed may attribute to the following reasons. 1) Nitrogen atoms of MB can form hydrogen bond with hydroxyl groups of other compounds **(Figure 6)** [52]. Thus, the hydroxyl group of Span 60 molecules interactwithamine groups of MB via hydrogen bonding interactions inside the core, and hence entrapped the drug within the vesicle.2) Moreover, the opposite charges would attract each other to hold the drug more efficiently as DCP is negatively charged and methylene blue has a positive charge.

Zeta potential of vesicles plays an important role in stability of niosomes values ranging between −41.7 and −58.4 mV. Generally charged niosomes possess a higher stability against aggregation and fusion than uncharged vesicles [53].

In our study, niosomes were negatively charged with zeta potentials between −39.5 and −80.9 mV and was more negatively charged on using DCP as stabilizer and thus gave sufficient electrostatic stabilization. However the zeta potential of noisome containing DCP at different cholesterol molar ratio to Span 60 showed a higher significant increase in comparison with its related formulations without DCP $p \leq 0.001$(N5 vs. N1& N6 vs. N2&N7 vs. N3&N8 vs. N4).

From the previous results we select N2 for further evaluation for the following reasons: has high EE (67.67 %), small particle size (328.3 nm), acceptable mono dispersity (0.497), and zeta potential (-45.6) in addition significantly highest in vitro release than other formulations.

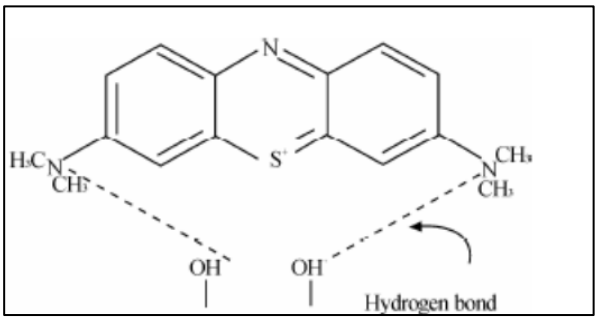

Figure (6): Hydrogen bonding between nitrogen atom of MB and hydroxyl groups

5.4. In vitro release of methylene blue from different gel formulations

The results of the in vitro release of MB from N2 niosomal dispersion in different gel bases are shown in **(Figure 7)**, statistically; there is a significant difference between release of the drug from HPMC 3 % gel base and from other formulation using ANOVA test at level of significance 0.05. On the other hand there is no significant difference between the release of the drug from pluronic 20 % gel and HEC 3 % gel formulations.

The mathematical assessment of the in-vitro release data of MB from niosomal gel is presented in **(Table 5)**. It was found that the drug release was in favor of Higuchi diffusion mechanism as confirmed by the values of correlation coefficients (r). The results indicate that incorporation of MB niosomes in HPMC 3% gel results in suitable and feasible release rate of MB.

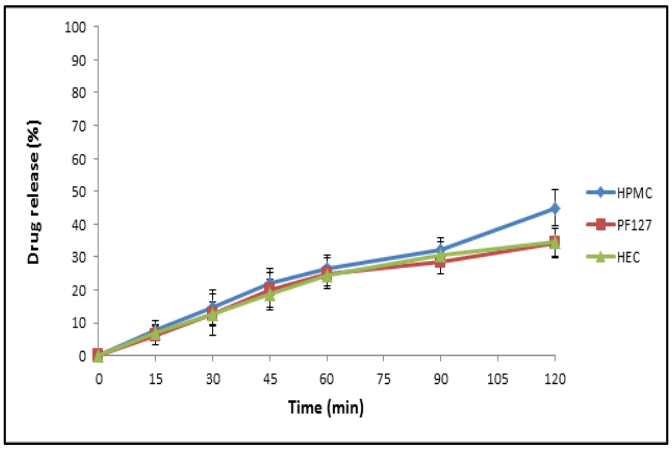

Figure 7: Effect of the gel type on the in vitro release of MB from noisome (N2) prepared by reverse phase evaporation technique using cholesterol: span 60 molar ratio (1:2).

5.5. Physical Stability

Visual appearance indicates that, the color of the niosomesN2 stored at 5±3 °C did not alter or change in the first and third months. However after 3 months, formation of dispersible precipitate was noticed. Zeta potential and %EE did not show any significant change, **(Table 6)**. The particle size significantly increased and PDI decreased after 3-month storage. This change might result from the aggregation of smaller niosomes. To prevent this, niosomes can be stored after lyophilisation.

5.6. Clinical evaluation

The study included 45 patients with acne vulgaris: 9 male and 36 female, with a mean ± (SD), age are 21.8 ± 2.47 years and mean ± (SD), disease duration was 3.52 ± 2.43 years. According to skin type, patients were divided into group III (n=12), group IV (n=23) and group V (n=10). According to Evaluator Global Severity Score (EGSS) of acne vulgaris (Eichenfield*et al.* [37], patients were divided into three grades of acne vulgaris mild degree (n=5), moderate (n=27) and severe (n=13), as shown in **(Table 7)**.

MB molecule has a great potential for application in PDT. It has an intensive ability to absorb light in the therapeutic window and effective photochemistry. Wainwright *et al.* [54] have found the photo bactericidal activity of MB against vancomycin resistant, and against methicillin resistant strains of Staphylococcus aurous.

According to a global grading scale [38] the improvement of inflammatory lesion in right side of the face (treated with IPL) was 8 patients showed an excellent improvement and 10 patients showed very good improvement while in left side of the face (treated with PDT) was 14 patients showed excellent improvement and 13 patients showed very good improvement, as shown in **(Table 8)**.

Table 5: kinetic data of in vitro release of N2 from different gel base

Mechanism of release	Zero order			First order			Diffusion			Best fitted model
Formulation	**R**	**K**	**t(1/2)**	**R**	**K**	**t(1/2)**	**R**	**K**	**t(1/2)**	
HPMC 3%	0.98	19.91	2.51	0.990	0.28	2.50	0.991	38.79	1.66	**Diffusion model**
PF127 20%	0.963	15.25	3.28	0.974	0.19	3.55	0.988	30.43	2.69	**Diffusion model**
HEC 3%	0.977	15.96	3.13	0.986	0.21	3.36	0.995	31.60	2.50	**Diffusion model**

Table 6: Stability test results of N2 niosomes kept at 5±3 °C

	N2 Niosomes		
	Initial	**1 month**	**3 month**
%EE	67.67±4.1	66.2 ± 3.8	65.7 ± 5.2
Particle size	328.3±8.2	348.5 ± 1.6	357.1± 5.4
Zeta potential	-45.6±2.3	-42.1 ± 2.53	-40.7± 6.1
PDI	0.497±0.001	0.405 ± 0.006	0.442± 0.003

Table 7: Clinical characteristic of studied patients (n =45)

Variable	Number of patients	Percent %
Sex		
Male	9	20%
Female	36	80%
Age		
Mean ± SD (Range)	21.8 ± 2.47 (17- 28y)	
Duration of disease (months)		
Mean ± SD (Range)	9.3 ± 6.1 (6– 25 months)	
Type of skin		
Type III	12	26.7 %
Type IV	23	51.1 %
Type V	10	22.2 %
Degree of acne vulgaris		
Mild	5	11.1%
Moderate	27	60 %
Severe	13	28.9 %

Table 8: Percentage of improvement of inflammatory lesions on both sides of the face

Variable	Right side		Left side	
	(n = 45)	**Percent %**	**(n = 45)**	**Percent %**
Excellent (75 - <100%)	8/45	17.8 %	14/45	31.1 %
Very good (50 - <75%)	10/45	22.2 %	13/45	28.9 %
Good (25 - <50%)	19/45	42.2 %	16/45	35.6 %
Poor (1 - < 25%)	8/45	17.8 %	2/45	4.4 %

Essam *et al* [55], reported that, treatment with IPL and NdYAG light sources may give rise to an improvements in inflammatory acne and acne scarring, but with minor effect for non-inflammatory (comedonal) acne.

PDT side revealed a significant tremendous improvement in inflammatory lesions ($P \leq 0.05$), comparing with improvement on the IPL side **(Table 9)**. This can be attributed to the characteristic properties of MB since it has a longer wavelength and higher intensity of absorption of light than coproporphyrin III and protoporphyrin IX produced by P. acne which resulted in complete destruction of the sebaceous glands. In addition to the antimicrobial effect of MB [56]. The combination of the previous properties of MB with the effect of IPL in reducing the vascularity of inflammatory lesions produced a favorable outcome.

In the present study, it was found that, there is significant improvement among patients treated with niosomal methylene blue gel photodynamic therapy **(Figure 8)** in comparison to the IPL therapy; improvement (> 50 %) was reported in 60 % of patients.

In the IPL side, the only side effect was a mild stinging occurring after the end of session and it was treated by icepacks, while in PDT side, mild pain during treatment session was the most common side effect reported in our study followed by slight transient erythema, also no serious adverse side effects were recorded.

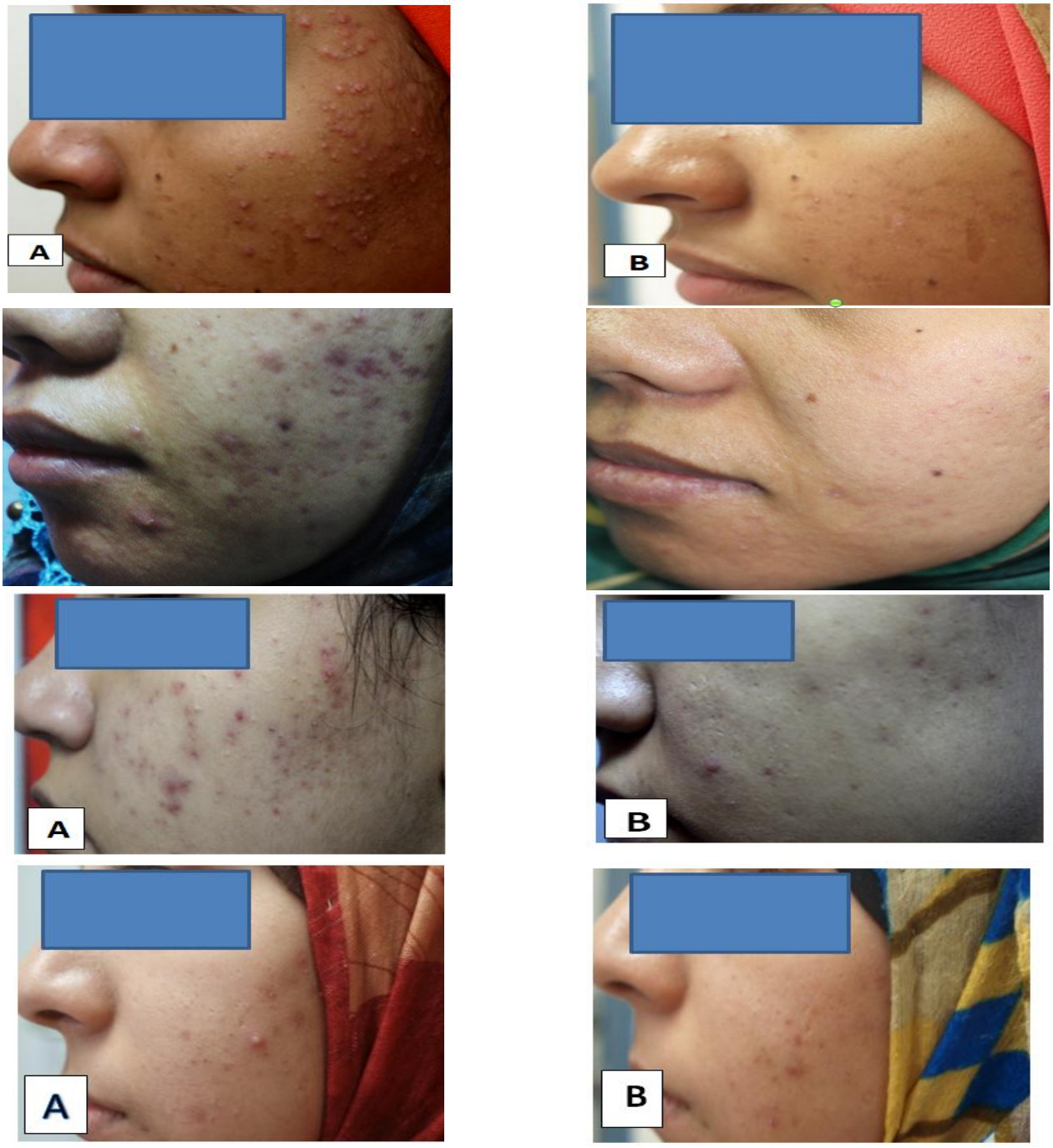

Figure 8: Inflammatory facial acne treated by MB mediated PDT A-before treatment, B-after treatment.

Table 9: A comparison of the percentage of improvement in acne lesion counts between the photodynamic therapy side and intense pulsed light.

Lesions count	The side treated with photodynamic therapy			The side treated with IPL			P value
	Before Treatment	After treatment	Percentage of reduction (%)	Before Treatment	After treatment	Percentage of reduction (%)	P ≤ 0.05*
Inflammatory acne lesions count	47.3±5.8	21.65±65	-54.32 ± 8.1	42.54 ± 7.3	28.6±4.7	-32.66±4.9	

*** P value ≤ 0.05 was considered statistically significant.**

6. Conclusion

Niosomes were successfully produced by reversed phase evaporation technique using different ratio of cholesterol: span 60. Formula N2 shows a significant higher release of MB than other formulae $p \leq 0.05$.Also the most favorable amount of MB could be used in niosomal preparation was 1000µg.The drug (MB) release from niosomal gel was favor of Higuchi diffusion mechanism. Moreover Formulation of niosomal MB in HPMC 3% gel gave rise to suitable release rate of MB. It is worthy to note that, PDT using MB as photosensitizer and IPL as light source open a window of effective treatment of acne lesion with minimum side effects, and provided a successful alternative remedy for patients in which topical or systemic medicines have failed or contraindicated

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