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Novel Probe Sonication Method for the Preparation of Meloxicam Bilosomes for Transdermal Delivery: Part One

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ABSTRACT

Probe sonication is a common method for the preparation of noisome, however almost all published bilosomes formulations are based on thin film hydration method which involves the usage of hazardous organic solvents. In this research article meloxicam bilosomes were successfully formulated using probe sonication method. Based on Box-Behnken design 17 different formulations were prepared with mean vesicle size ranged from 147.933 ± 6.87 nm to 308.6 ± 109.5 nm, zeta potential ranged from -17.16 ± 3.9 to -27.16 ± 7.47 and with entrapment efficiency % ranged from 9.3 ± 1.22% to 94.8 ± 1.76%. In vitro release studies show sustained release patterns which are characteristic for bilosomes formulations. In conclusion probe sonication method is an efficient and ecologically friendly method for the preparation of meloxicam bilosomes intended for transdermal delivery.

Keywords: Probe sonicator, Bilosomes, Transdermal, Meloxicam, Box-Behnken

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INTRODUCTION

The human's skin which represents about 16% of the total body weight [1] is considered as the largest organ of the body [2] with main duty to protect the body against invading foreign bodies [3]. The outermost layer of the skin knowns as stratum corneum is considered as the main barrier against skin penetration due to its low permeability [4]. Transdermal drug delivery has gained interest in the last years with estimated market value of transdermal products of 9.5 billion dollars a year worldwide till 2017 [5]. The main challenge for drug delivery across the skin is how to pass through the stratum corneum; several approaches have been tested for this purpose including physical, chemical, and vesicles-based methods [6].

Physical methods including different approaches such as microneedles [7-10], jet injector, laser ablation, electroporation [11-14], sonophoresis iontophoresis and magnetophoresis [15-18]. Chemical methods involve the usage of chemical compounds which act as penetration enhancers such as ethanol [19-22].

Vesicles-based methods involve the employment of vesicle carriers to enhance skin penetration such as liposomes niosomes and bilosomes [23-25].

Bilosomes were firstly described by Conacher [26] are bilayer vesicles like niosomes in structure but with the inclusion of bile salts in the bilayer [27]. Bilosomes were firstly tested for transdermal delivery by Al-Mahallawi in a research article that concluded the potential uses of these vesicles carrier to enhance skin penetration of tenoxicam [28].

Inflammation considered as the alarm system of the body against tissue injury however, prolonged inflammation can result in tissue damage [29], inflammation involves the activation of cyclooxygenase (COX) enzymes which act as inflammatory mediators [30]. Non-steroidal anti-inflammatory drugs (NSAIDs) which act through the inhibition of COX enzymes are among the most prescribed medicines due to their wide variety of uses including anti-inflammatory, anti-pyretic and analgesic activities [31].

Meloxicam which was approved by the FDA in 2000 as 7.5 mg tablet [32] is a non-steroidal anti-inflammatory drug belong to the oxicam group and has shown selective activity for inhibition of COX-2 enzyme over COX-1 enzyme. Despite its safety, meloxicam has the unwanted gastrointestinal adverse effects of NSAIDs which make transdermal delivery of it favorable over oral route however due to its low water solubility there are obstacles against successful transdermal delivery, different approaches have been employed for enhancing

meloxicam transdermal delivery [33-36]. However, based on our knowledge bilosomes have never been employed for enhancing transdermal delivery of meloxicam.

The aim of part one of these researches is to test if probe sonication method which is employed for the preparation of niosomes can be employed for bilosomes preparation. Till now almost all bilosomes formulations are prepared by using thin film hydration method which involves the usage of hazardous organic solvents. Based on our knowledge this part is the first to describe the preparation of bilosomes using probe sonication method.

MATERIALS AND METHODS

Meloxicam, Sodium Deoxy Cholate (SDC), Cholesterol, Sorbitan monostearate(Span® 60), Absolute ethanol 99.8%.

Preparation of Meloxicam Bilosomes

In a beaker, a constant amount of Span® 60 (420 mg) was mixed with different amounts of meloxicam, cholesterol and sodium deoxy cholate as shown in Table 2. To the above mixture 20 ml of distilled water was added and the resultant dispersion was homogenized with a homogenizer running at 3000 RPM for 5 minutes. After that the resultant dispersion was subjected to probe sonication for 5 minutes (50 seconds on and 10 seconds off with 30% amplitude) (Q500 QSONICA Sonicator, Qsonica, LLc. USA). Finally, the resultant milky dispersion was stored at 4°C overnight to allow vesicles to mature and remained at the refrigerator until further characterization.

In vitro characterization

Light Microscopic Study

To assure the formation of vesicles, 1ml of different dispersions were tested under the light microscope using different magnifications (10X, 40X, and oil immersion 100X).

Determination of vesicles size, polydispersity index and zeta potential

1 ml of each formulation was diluted with 10 ml of distilled water to reach faint opalescence and tested for vesicles size, Poly Dispersity Index (PDI) and zeta potential using Zetasizer Ultra. The used cuvettes were made of quartz and the instrument refractive index was adjusted at 1.33.

Determination of entrapment efficiency %

To determine the entrapment efficiency %(EE %) a method reported in [37] was used. Briefly, 1 ml of each bilosomes formulations was diluted with 9 ml of absolute ethanol 99.8% then actual drug content was determined spectrophotometrically using UV absorbance (CARY 100 Conc UV-Visible spectrophotometer, Varian, Australia) read at 354 nm wavelength using calibration curve of meloxicam in absolute ethanol 99.8% (R2 = 0.997 n = 3) after suitable dilutions. Then the entrapped drug was determined through taking 1 ml of each bilosomes formulations and subjected to centrifuge using cold centrifuge (eppendrof centrifuge 5417 R, Eppendrof AG, Germany) running at 9000 RPM at 4°C temperature for 90 minutes, the supernatant was discarded and the precipitates were dissolved in 10 ml absolute ethanol 99.8% with the aid of sonication using bath sonicator operates at 56°C temperature for 5 minutes then entrapped drug was determined spectrophotometrically as above. Finally, the entrapment efficiency of each formulation was calculated using the equation below

$$Entrapment\ efficiency\ \% \frac{Entrapped\ drug}{Actual\ drug\ content} \times 100$$

Studying the influence of different formulation variables using Box-Behnken design

Box-Behnken design was employed using Design-Expert® version 13.0.5.0 software (Stat Ease, USA). The three independent variables were: (X1: Meloxicam dose), (X2: Cholesterol amount) and (X3: SDC amount). The dependent variables or responses were (Y1: Vesicle size), (Y2: Zeta potential), (Y3: PDI) and (Y4: Entrapment efficiency%) as shown in table 1. 17 formulations with 5 center points of meloxicam bilosomes were prepared based on the above software as shown in table 2.

Abbreviations: SDC (Sodium deoxycholate), PDI (Polydispersity index), EE% (Entrapment efficiency %), a: experiments were done as triplicate with results represent mean ± standard deviation.

Optimization of meloxicam bilosomes

Design-Expert® version 13.0.5.0 software (Stat Ease, USA) was used to select optimal bilosomes formulations for in vitro drug release study by applying the desirability function. The selected optimization criteria were to get formulas with least vesicle size and polydispersity index and with highest entrapment efficiency and zeta potential as absolute values.

In vitro drug release study

In vitro drug release was performed for the selected bilosomal formulations. Dialysis bag method was used to determine the amount of drug released after 6 hours, in brief an equivalent amount to 1.5 milligrams of meloxicam was taken from the selected bilosomes formulation and poured in dialysis membrane (which were soaked overnight in release media) (M.wt 8000-14000, Special products laboratory, USA) then the dialysis bags were placed in type two dissolution apparatus (paddle type) (RC-6 Dissolution tester, Faithful, China).The release media was 250 ml Phosphate puffer saline (PBS) PH 7.4 solution to achieve sink condition. The apparatus temperature was 37±0.2 and the paddle rotation speed was 100 Round Per Minute (RPM).At predetermine time (1, 2, 3, 4, 5 and 6 hours) three milliliters samples are withdrawn and replaced by fresh PBS solution to maintain sink condition. The withdrawal samples were tested for meloxicam amount spectrophotometrically using UV-Visible spectrophotometry (CARY 100 Conc

Table 1: Dependents and independents variables used in Box-Behnken design.

Dependent variables	Levels
X1: Meloxicam dose	5 mg, 10 mg, and 15 mg
X2: Cholesterol amount	60 mg, 180 mg, and 300 mg
X3: SDC amount	5 mg, 10 mg, and 15 mg
Y1: Vesicle size	Minimized
Y2: Zeta potential	Maximized
Y3: PDI	Minimized
Y4: Entrapment efficiency %	Maximized

Table 2: Formulations of meloxicam bilosomes done using Box-Behnken design.

Formula	Meloxicam dose	Cholesterol amount	SDC amount	Vesicle size[a] nm	Zeta potential[a]	PDI[a]	EE[a] %
F1	5	60	10	237.933 ± 75.43	-19.8 ± 1.31	0.335 ± 0.133	13.9 ± 1.57
F2	15	60	10	263.8 ± 51.84	-22.39 ± 8.61	0.268 ± 0.093	94.8 ± 1.76
F3	5	300	10	147.933 ± 6.87	-21.7 ± 5.77	0.335 ± 0.008	9.3 ± 1.22
F4	15	300	10	184.533 ± 16.59	-24.05 ±10.62	0.278 ± 0.024	69 ± 2.91
F5	5	180	5	190.733 ± 19.42	-17.16 ± 3.9	0.255 ± 0.065	17.8 ± 6.27
F6	15	180	5	187.767 ± 7.31	-18.3 ± 2.16	0.244 ± 0.018	62.3 ± 1.95
F7	5	180	15	187.3 ± 12.46	-27.16 ± 7.47	0.251 ± 0.036	23 ± 9.59
F8	15	180	15	239.633 ± 50.7	-25.17 ± 8.95	0.271 ± 0.008	65.8 ± 1.63
F9	10	60	5	308.6 ± 109.5	-18.2 ± 2.95	0.259 ± 0.058	34.8 ± 2.95
F10	10	300	5	206.3 ± 29.52	-17.3 ± 0.72	0.223 ± 0.094	48.9 ± 1.37
F11	10	60	15	283 ± 81.81	-25.87 ± 7.51	0.236 ± 0.017	23.1 ± 8.15
F12	10	300	15	226.8 ± 28.15	-24.89 ± 8.61	0.25 ± 0.062	35.9 ± 1.7
F13	10	180	10	229 ± 19.67	-24.2 ± 9.1	0.276 ± 0.016	36.4 ± 6.39
F14	10	180	10	228.7 ± 34.21	-24.6 ± 9.76	0.281 ± 0.012	42.1 ± 7.48
F15	10	180	10	240.733 ± 32.39	-24.3 ± 9.55	0.245 ± 0.026	36.2 ± 1.6
F16	10	180	10	254.433 ± 75.38	-23.6 ± 7.1	0.246 ± 0.009	33.8 ± 4.09
F17	10	180	10	217.033 ± 37.72	-22.4 ± 8.61	0.219 ± 0.033	42.9 ± 1.13

UV-Visible spectrophotometer, Varian, Australia) reading the concentration at the λ max of meloxicam in PBS PH 7.4 which is 362 nm and by using the calibration curve equation of it (R2 = 0.998, n = 3). The release experiments were done as triplicate [38].

Release Kinetic Modelling

The obtained in-vitro release data from different bilosomes formulations were fitted to different mathematical equations using DD-solver and Microsoft excel® 2016 program for determining the mechanism and kinetic of meloxicam release from bilosomes formulations [39]. The used kinetic models were: zero order release kinetic model (cumulative percentage drug release vs. time), first order release kinetic model (Log cumulative percentage drug retained vs. time), Higuchi release kinetic model (cumulative percentage drug release vs. cubic root of time), and finally Korsmeyer-Peppas release kinetic model (Log cumulative percentage drug release vs. Log time), model with the highest correlation coefficient was selected to be the best fitted model [40].

Selection of the optimal bilosomes formula.

Depending on the amount of meloxicam released after 6 hours, the formula that shows the highest amount of drug release was chosen as best formula to be studied further in part two of this research.

RESULTS AND DISCUSSION

Analysis of factorial design

Today, the use of experimental designs is a common method for analyzing the effect of different variables on the characteristics of the drug delivery system under study, theses variables can affect the properties of the final dosage form [41]. In this study all the selected independent variables and their levels are chosen based on preliminary data which are not shown here. In all responses studied, adequate precision value greater than 4 (the desired value) was observed indicating that the chosen model for each response can be used effectively [42].As shown in table 3 the predicted R2 values for all responses except PDI are in reasonable agreement with the adjusted R2 as the differences is less than 0.2. The negative predicted R2 of PDI implies that the overall mean is a better predictor for the response.

Light microscope study

Results of light microscope are shown in figure 1 which indicates the formation of vesicles that support the use of probe sonication for the preparation of bilosomes.

Effect of formulation variables on vesicle size

In transdermal delivery dosage form vesicles or particles size plays an important role in the penetration of vesicles or particles across the skin, that the smallest vesicles or particles penetrate the skin deeper and hence

enhance transdermal delivery. The prepared bilosomes vesicles were in nano sized range with a mean diameter fluctuated from 147.933 ± 6.87 nm to 308.6 ± 109.5 nm. The effects of independent parameters on vesicle size are shown as 3D plots in figure 2. ANOVA study was used to determine the significant of independent variables on vesicle size, results indicate that both meloxicam dose (X1) and cholesterol amount (X2) have significant effects on vesicle size ($P < 0.05$) whereas Sodium deoxycholate amount (X3) has a non-significant effect on vesicle size ($P > 0.05$).Regarding the effect of meloxicam dose (X1) on vesicle size, meloxicam dose has a significant positive effect on vesicle size ($P = 0.0193$) those when increasing the dose, the vesicle size increased, this can be due to increasing the amount of entrapped drug when increasing meloxicam dose, that more entrapped drug resulted in larger vesicles hence increasing the vesicle size, similar results were obtained in the literature [43,44].Regarding the effect of cholesterol amount (X2) on vesicles size, increasing cholesterol amounts paradoxically resulted in smaller vesicles those cholesterol has a significant negative effect on vesicle size ($P< 0.0001$) this can be interrupted as the incorporation of cholesterol in bilosomes increase the bilayer hydrophobicity which resulted in decreasing surface energy and vesicles size. Similar results were obtained for the effect of cholesterol on decreasing the vesicles size of niosomes prepared from Span® 60 [45].

Effect of formulation variables on zeta potential.

Zeta potential can be defined as a measurement of the total surface charge of vesicles; it can be used as an indication for the stability of the system, in general large value of zeta potential as an absolute number indicates a more stable system [46].

In this research the values of zeta potential were varied from -17.16 ± 3.9 to -27.16 ± 7.47 indicating that most formulations have sufficient surface charge. The negative charge of all formulation resulted from the anionic nature of the bile salt sodium deoxy cholate (SDC).ANOVA study indicating that only the amounts of SDC (X3) has significant effect on the value of zeta potential ($P < 0.05$). Regarding the effect of SDC on zeta potential, a significant positive effect ($P < 0.0001$).As shown in figure 3, increasing the amount of SDC resulted in increasing the absolute value of zeta potential, this is due to that bile salts act as membrane stabilizers that added charge to the membrane surface and hence increasing the stability, similar results were obtained in the literature [47].

Effects of formulation variables on PDI.

Polydispersity index (PDI) is a measure of the homogeneity of the formulations. A formulation with a PDI value close to 0 indicates a uniform population while those with a PDI value close to 1 indicates highly polydisperse system [48]. In our work PDI values ranged from 0.219 ± 0.033 to 0.335 ± 0.133 indicates monodisperse systems. ANOVA study indicates that none of the independent variables has a significant effect on the values of PDI. Similar results were reported by literature [49].

Effects of formulation variables on entrapment efficiency %.

Entrapment efficiency % of the prepared bilosomes formulation was ranged from 9.3 ± 1.22% to 94.8 ± 1.76%. regarding ANOVA study only meloxicam dose (X1) has a significant effect on the entrapment efficiency % ($P < 0.05$), neither cholesterol (X2) nor SDC (X3) amounts have a significant effect on entrapment efficiency %.The 3D plots of the effects of independent variables on entrapment efficiency are shown in figure 4.Regarding meloxicam dose (X1) a significant positive effect was observed with ($P < 0.0001$), this may be due to that increasing meloxicam dose resulted in increasing media saturation with meloxicam, those forcing it to be entrapped within bilosomes, similar results were reported in the literature [50].

Only meloxicam dose has a significant positive effect on

Table 3: Output data of the Box- Behnken design.

Responses	R^2	Adjusted R^2	Predictued R^2	Adequate precision	Significant factors
Vesicle size	0.9527	0.892	0.7017	15.1829	X1, X2
Zeta potential	0.8239	0.7833	0.7093	12.6968	X3
PDI	0.7046	0.3247	-1.5097	4.7853	None
Entrapment efficiency %	0.8267	0.7867	0.6483	12.2534	X1

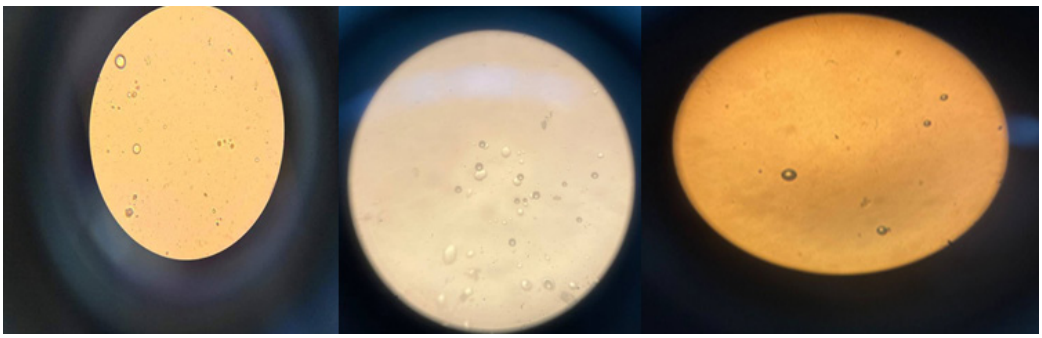

Figure 1: Light microscope study, A under 100X oil immersion, B under 40X, and C under 10 X.

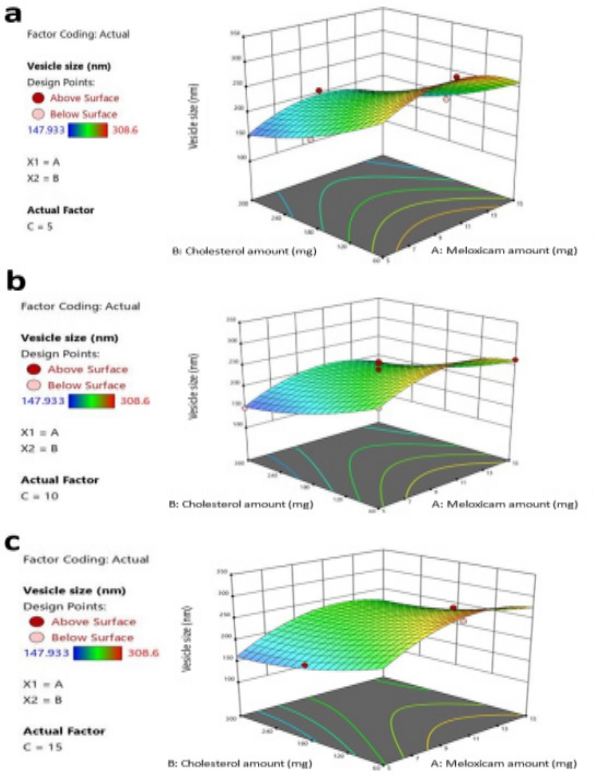

Figure 2: Response surface 3D plots of independent variables effects on vesicles size. (As shown that both meloxicam dose and cholesterol amount have significant effect on vesicle size whereas increasing SDC amount has no significant effect on vesicle size), (a- 5 mg SDC, b- 10 mg SDC, c-15 mg SDC).

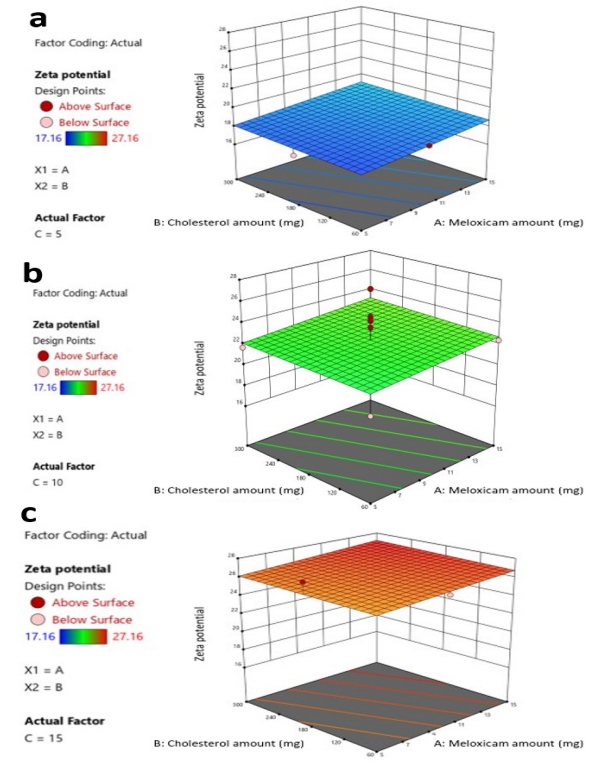

Figure 3: Response surface 3D plots of independent variables effects on zeta potential. (As the amount of SDC increased from 5 mg to 10 mg to 15 mg, the zeta potential values increased significantly) , (a- 5 mg SDC, b- 10 mg SDC, c-15 mg SDC).

EE %, increasing meloxicam dose from 5 mg to 10 mg to 15 mg results in statistically significant increase in EE %, (a- 5 mg SDC, b- 10 mg SDC, c-15 mg SDC).

Selection of the optimal bilosomes formulations

By applying the desirability function design experts was used to select optimal bilosomes formulations to be studied further. The selected bilosomes formulations with their desirability values are shown in table 4, whereas table 5 compared predicted vs actual dependent variables for the selected bilosomes formulations.

In vitro drug release

The release profiles of the selected bilosomes formulation are shown in figure 5. F2 shows the highest release percentage after six hours reaching 100 % release of the entrapped meloxicam. The release profiles for all tested formulations show a sustained drug release which can be interrupted as the bilosomes just like other colloidal vesicles systems act as drug reservoir that release the entrapped drug in sustained fashion, similar results were obtained in the literature [51]. To study the effects of different formulation variables on the percentage of release after six hours, F2 and F4 release was used to study the influence of cholesterol amount on the percentage of drug release since these two bilosomal formulas differ only in the amount of cholesterol F2 contains 60 mg of cholesterol while F4 contains 180 mg of cholesterol. While F6 and F8 release profiles were used to study the effect of sodium deoxycholate on the percentage of drug release after six hours as these formulas only differ in the amount of SDC they contain F6 contains five mg of SDC while F8 contains 15 mg of SDC. Regarding the effects of cholesterol amount on the amount of drug release, increasing cholesterol amount

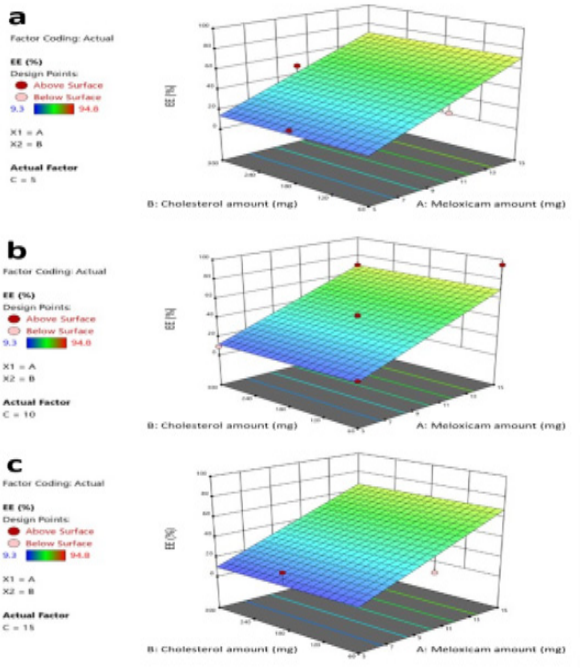

Figure 4: Response surface 3D plots of independent variables effects on entrapment efficiency %.

form 60 mg in F2 to 180 mg in F4 results in decreasing the amount of drug release, that cholesterol has a negative effect on the release of drug from bilosomes. This effect may be due to when increasing cholesterol amounts the wall of bilosomes becomes stiff and this hindered drug release from bilosomes, similar result was obtained in the literature. Regarding SDC amount effects on the amount of drug release, increasing SDC amount form five mg in F6 to 15mg in F8 results in increasing the amount of drug release, those SDC has a positive effect on the release of drug from bilosomes. This can be due to that bile salts act to increase bilosomes wall flexibility which facilitates release of the entrapped drug, similar result was obtained in the literature [52].

Release kinetic modelling

Different mathematical models were used to simulate the release of meloxicam from different bilosomes formulations. The values of release constants and regression coefficients are listed in table 6. The best describing release model is based on the highest R2 values, from the above table the Korsmeyer-Peppas model has the highest R2 values for all tested formulations those it was chosen as the best fit model for describing the mechanism of meloxicam release from bilosomes formulation, similar results were obtained in the literature for the release of drug from bilosomes (52). Regarding the exponent n values, all formulas except F4 showed value larger than 0.5 meaning that the drug transport mechanism is non-Fickian anomalous transport (that both diffusion and erosion is involved in drug release mechanism), F4 with n value less than 0.5 shows quasi Fickian diffusion drug transport. Similar results regarding the exponent values were shown in the literature [53].

Selection of the optimal bilosomes formula

F2 which shows the highest release amount after 6 hours was chosen for further studies in part two of this research mainly for ex-vivo permeation and *in-vivo* studies together with transmission electron microscope images and finally stability studies.

Table 4: Desirability of selected bilosomes formulations.

Formula	Desirability
F2	0.488
F4	0.685
F6	0.446
F8	0.673
F12	0.534

Table 5: Predicted vs actual dependent variables of selected bilosomes formulations.

Formula	Predicted / actual VS	Predicted / actual ZP	Predicted / actual PDI	Predicted / actual EE%
F2	260.817 / 263.8	1.0146494	0.289 / 0.268	69.513 / 94.8
F4	184.242 / 184.533	0.962079	0.291 / 0.278	68.638 / 69
F6	196.096 / 187.767	1.0334426	0.23 / 0.244	71.076 / 62.3
F8	234.579 / 239.633	1.0705205	0.252 / 0.271	67.076 / 65.8
F12	232.146 / 226.8	1.0704299	0.256 / 0.25	38.151 / 35.9

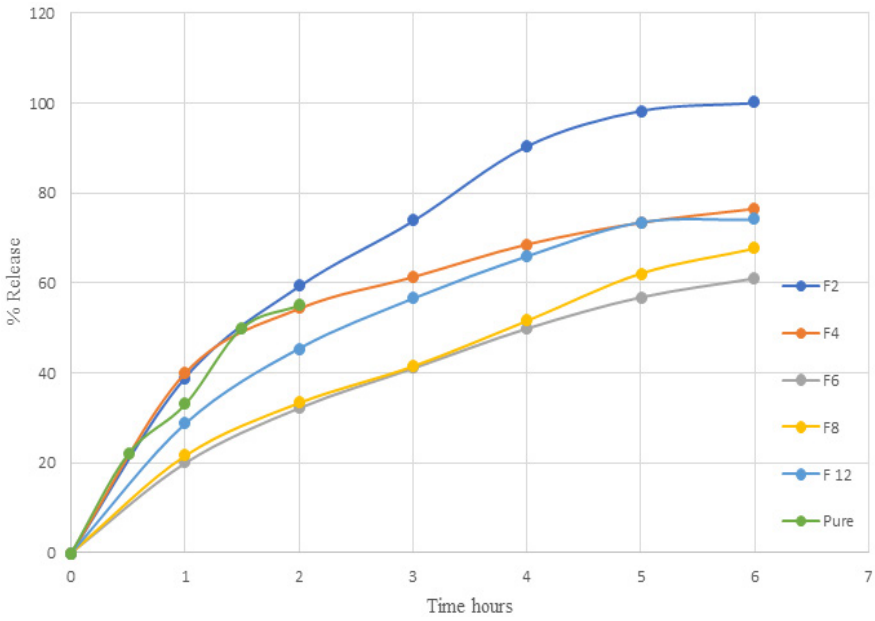

Figure 5: In-vitro drug release from selected bilosomes formulations, F2 formula reaches 100 % drug release after 6 hours.

Table 6: release kinetics values summary.

Formula	Zero order		First order		Higuchi model		Korsmeyer-Peppas model		
	K_0	R^2	K_1	R^2	K_H	R^2	K_{KP}	n	R^2
F2	20.123	0.8215	0.502	0.9858	42.653	0.9914	41.65	0.517	0.9917
F4	15.743	0.6049	0.316	0.9204	33.883	0.9688	41.541	0.351	0.9983
F6	12.473	0.9337	0.19	0.9915	26.1	0.9775	20.824	0.661	0.998
F8	11.611	0.9028	0.172	0.9862	24.407	0.9878	20.965	31.679	0.9982
F12	14.993	0.8065	0.271	0.9822	31.825	0.9914	0.609	0.503	0.9914

CONCLUSION

In this part of our research probe sonicator was successfully employed for the preparation of meloxicam bilosomes. This method represents an ecological friendly and simple method when compared to thin film hydration which is the one most widely used method for the preparation of bilosomes. Based on our knowledge this part is the first published article that discusses the preparation of bilosomes using novel probe sonication methods. Future studies will be conducted to study the permeation and other testes of the best selected meloxicam bilosomes formulation.

Author Contributions

Authors contribute to this research equally.

Conflicts of Interest

The authors report no conflicts of interest in this work.

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