Solubilization of Hydrophobic Drugs Clozapine and Oxcarbazepine in the Lower and Higher Molecular Weight Pluronic Mixed Micelles-A Physicochemical, *In Vitro* Release and *In Vitro* Anti-oxidant Study

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Abstract

In this work, we studied the solubilization capacity of the hydrophobic drugs Clozapine (CLZ) and Oxcarbazepine (OXC) in mixed micelles of low molecular weight pluronics (L64 and L35) and high molecular weight pluronics (P84, F127, F68 and P123) using UV-visible spectroscopy. The drug solubility, drug loading efficiency, the micelle–water partition coefficient (*P*), and Gibbs free energy ($\Delta G°$) of solubilization were measured. From the obtained results, we concluded that the highest solubility of CLZ was observed in binary mixture of L64/P84 (1:4) due to the hydrophobic nature of these pluronics. In the case of OXC, the highest solubility was observed in L64/F127(1:4) because the drug solubilized both in the core and the corona region of F127 since OXC contains a carboxamide polar moiety. Fluorescence spectroscopy indicated that the critical micelle concentration (*cmc*) decreased with the formation of mixed micelles as compared to pure individual micelles. From DLS measurements, it was observed that there was an increase in size of mixed micelles from mixed micelles to drug (CLZ and OXC) loaded mixed micelles. *In vitro* release study of CLZ showed that as the amount of P84 in binary mixed micelles decreases, the release was faster. The formulations were also screened for antioxidant activity by four different methods. The antioxidant activity was significantly increased in the binary mixture of the pluronic solutions, indicated that pluronic formulations can be considered as an effective drug delivery candidate in the management of diseases that affect the central nervous system.

Keywords: Hydrophobic Drugs; Solubilization; Antioxidant Assays; *In vitro* Release, Dynamic Light Scattering

1.0 Introduction

Reactive oxygen species (ROS) are continuously generated when oxygen is consumed for different physiological processes. ROS are comprised of free radicals and are generated under normal physiological conditions and removed by the body's antioxidant defense mechanism.[1] An imbalance between ROS and the antioxidant defense mechanism leads to oxidative stress. ROS can initiate the oxidative damage to amines, proteins, lipids, nucleic acid etc.[2] If these radicals are not scavenged properly, they can cause damage leading to abnormal conditions. Many diseases are the manifestation of reactive oxygen species like cancer, neuropathic pain, heart diseases, stroke, and various mental disorders like schizophrenia.[3-5]

Schizophrenia and epilepsy are among the most severe and chronic forms of mental disorders but their etiopathogenesis illness is not clear.[5] Various evidences suggest that oxidative stress, lipid peroxidation, and nitrosative stress contribute to the pathogenesis of schizophrenia and epilepsy.[6] Clozapine [8-chloro-11-(4-methyl-1-piperazinyl)-5*H*-dibenzodiazepine (CLZ)] is the drug of choice for the treatment of refractory schizophrenia and for treatment-resistant patients.[7-9] Oxcarbazepine (OXC), chemically 10, 11-dihydro-10-oxo-5H-dibenz[b,f]azepine-5-carboxamide, an antiepileptic drug has been used alone or in combination with other antiepileptic drugs as treatment therapy for partial seizures in adults and children.[10-12] To enhance the adsorption and bioavailability of hydrophobic drugs, many different approaches have been implied.[13,14] Among these approaches, amphiphilic copolymers have gained attention due to its capability to enhance water solubility of poorly water soluble drugs. One specific class of amphiphilic triblock copolymers, polyethylene oxide (PEO)-polypropylene oxide (PPO)-polyethylene oxide (PEO) block copolymers known as pluronics. Pluronics have been proved to be potential drug carrier for controlled drug release and moreover can encapsulate the hydrophobic drugs. Pluronics tri-block copolymers are US FDA approved commercially available non-ionic surface active agents.[15-19] Pluronic micelles are able to accommodate the hydrophobic drugs in the hydrophobic core made up of a PPO region and some polar drugs may be solubilized in the PEO corona region.[20-23] Poor drug loading capacity, large size distribution and stability issues are often associated with micelles formed from a single pluronic. However, mixed micelles composed of different pluronics show synergistic properties compared to those composed of a single pluronic, which include improved micelle

stability and increased solubilization capacity.[24-27] The anticancer drug doxorubicin in the mixed micelles made up of pluronics L61 and F127 was the first anti-cancer drug containing micellar formulation to reach the clinic and is currently undergoing Phase II clinical trials.[28-30] There are a number of studies in which pluronics serve as drug delivery carriers.

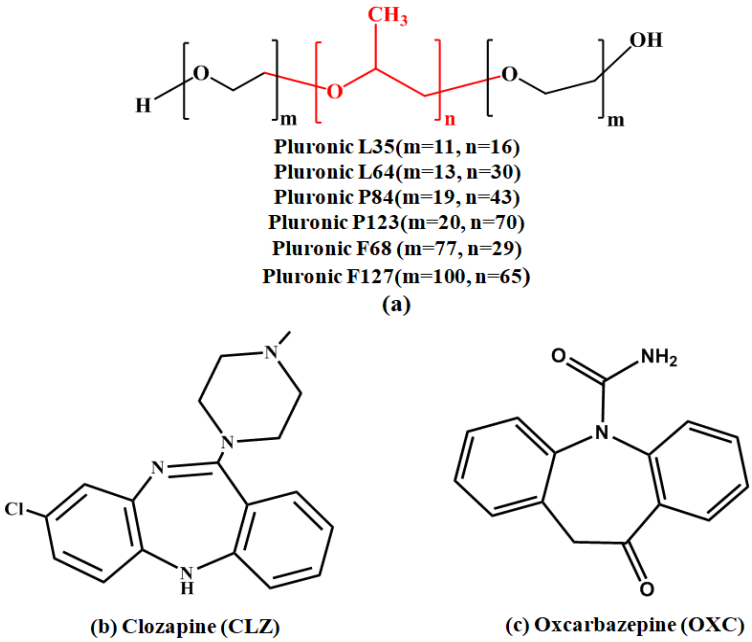
Dash *et al.* reported the interaction and mixed micellization behaviour of methylparaben and propylparaben with pluronic F127 and P123.[31] The UV studies revealed that mixed micelles of F127 and P123 with drug parabens showed intermediate spectral behaviour. When the drugs methylparaben and propylparaben were combined with the mixed pluronic system, quenching of a static nature was observed. These results indicated that binding between methylparaben and mixed micelles was stronger as compared to propylparaben and mixed micelles. Furthermore, Dash and co-workers reported the solubilization of the drug Ciprofloxacin in mixed micelles of Pluronic F108 and Pluronic L81 by using different techniques.[32] The values of the partition coefficient confirmed that there was higher solubility in pluronic mixed micelles as compared to pure pluronic micelles. DLS measurements evidenced that the pluronic mixture binds the drug better than single pluronics.

In this work, the solubilization behaviour of oxcarbazepine (OXC) and clozapine (CLZ) in binary mixtures of lower molecular weight pluronics *viz.* L64 ($PEO_{13}PPO_{30}PEO_{13}$) and L35 ($PEO_{11}PPO_{16}PEO_{11}$) with relatively higher molecular weight pluronics *viz.* P84 ($PEO_{19}PPO_{43}PEO_{19}$), F127 ($PEO_{100}PPO_{65}PEO_{100}$), F68 ($PEO_{77}PPO_{29}PEO_{77}$) and P123 ($PEO_{20}PPO_{70}PEO_{20}$) has been adjudged by UV-visible measurements. Critical micelle concentration (*cmc*) of mixed micelles was calculated using fluorescence measurements. Dynamic light scattering (DLS) measurements have been used to determine the hydrodynamic diameter (D_h) of the loaded and unloaded pluronic mixed micelles. *In vitro* drug release studies of the best solubilized formulation of binary mixed micellar systems containing CLZ were performed to determine the drug release behaviour of the drug from the mixed micellar systems. Antioxidant activity of different formulations was determined by various antioxidant assays.

2.0 Materials and Methods

2.1 Materials

Pluronics P84, P123, F127, F68, L64, L35 and the drugs oxcarbazepine (OXC) and clozapine (CLZ) were acquired from Sigma Aldrich having a percentage purity > 95%. All the materials were used without any further purification. The molecular structures of all the above mentioned chemicals are shown in **Scheme 1**. The molecular weights of different pluronics are tabulated in **Table S1** (supplementary information).



Scheme 1: Molecular structure of **(a)** Pluronics **(b)** Clozapine (CLZ) **(c)** Oxcarbazepine (OXC)

2.2 Methods

2.2.1. Solubilization Study of CLZ and OXC in binary mixtures of pluronics

The direct dissolution method was adopted for studying the solubilization of CLZ and OXC in binary mixtures of different pluronics. Pre-weighed amount of different pluronics were dissolved in 5 mL of double distilled water and then excess quantity of the drug was added to mixed pluronics solutions. These solutions were stirred at 37±1°C for 24 h and subsequently the samples were filtered using a Millipore filter (0.20 µm) to remove unloaded drugs. The concentration of the solubilized CLZ and OXC in different pluronic mixed micelles was determined using absorbance measurements performed with a Shimadzu (UV-1800) UV-visible double beam spectrophotometer. The stock

solution (2 mM of CLZ and OXC) was prepared in methanol and further diluted with water for obtaining the desired concentration range of 0.01 mM to 0.1 mM. The λ_{max} of CLZ and OXC was determined to be 292 nm and 255 nm respectively. The molar absorption coefficient of CLZ and OXC were calculated to be 10.54 L $mol^{-1}cm^{-1}$ and 8.81 L $mol^{-1}cm^{-1}$ respectively, using the slope of the calibration curve (absorbance versus concentration of CLZ and OXC). From this molar absorption coefficient, the concentration of loaded amount of CLZ and OXC was computed.

2.2.2 Critical Micelle Concentration (*cmc*) Measurements

The *cmc* values of the mixed micelles of different pluronics were determined at 37°C using a Hitachi F-4600 fluorescence spectrophotometer. Pyrene was used as hydrophobic probe. The temperature was controlled using an automated thermostat from ORBIT RS with an accuracy of $\pm$ 0.1°C.

2.2.3 DLS measurements

2.2.3.1 Size measurement- The hydrodynamic diameter (D_h) of loaded and unloaded mixed micelles of different pluronics were determined using a Malvern Zetasizer Nanoseries Nano-ZS instrument equipped with He-Ne laser (λ = 632.8nm) at 37°C at a scattering angle of 173°. Samples were prepared in double distilled water and prior to DLS measurements, samples were sonicated for 10 minutes and stirred overnight to generate monodisperse micelles.

2.2.3.2 **Measurement of Critical micelle temperature (*cmt*) -** Critical micelle temperature (*cmt*) can be measured with DLS. The derived count rate was monitored at a back-scattering angle of detection, at 2°C increments at different temperature range for different mixtures of pluronics in order to determine the *cmt*.

2.2.4. *In vitro* drug release

To study the *in vitro* drug release of CLZ drug from mixed micelles of different pluronics, the dialysis method was employed. 2 mL of CLZ loaded mixed micelles was placed in dialysis tubing (Himedia, Molecular weight cut-off 8000 kDa) and subsequently immersed in 100 mL of a buffered solution (pH= 7.4) which act as release medium to provide sink condition. The dialysis system was maintained at 37 $\pm$ 0.2°C with constant stirring at 120 rpm. Samples were withdrawn periodically and replenished with the same

volume of fresh release medium. Withdrawn samples were filtered with a 0.45µm filter and assayed spectrophotometrically.

2.2.5 DPPH free radical scavenging activity

To investigate the antioxidant activity of pure drugs CLZ and OXC, CLZ unloaded and loaded L64/P84 1:4 (%w/v) and OXC unloaded and loaded L64/F127 1:4 (%w/v) mixed micellar solution, 2,2-diphenyl-1-picryl-hydrazil free radical ($DPPH^{\bullet}$) scavenging assay was performed. 1 mL of solution was added to 3 mL of pure CLZ and OXC solution; empty mixed micellar solution of L64/P84 1:4 (% w/v) and L64/F127 1:4 (% w/v); CLZ and OXC loaded mixed micellar solution of L64/P84 1:4 (% w/v) and L64/F127 1:4 (% w/v) respectively at different concentrations of 200-1000 µg/mL. The absorbance of $DPPH^{\bullet}$ was evaluated at 517 nm after 30 min. The decrease in absorbance of reaction mixture depicts the increase in antioxidant activity.[33]

2.2.6 Metal chelating activity

The method of Dinis *et al.*[34] was used to evaluate the ferrous ions chelating of pure drugs CLZ and OXC, CLZ unloaded and loaded L64/P84 1:4 (%w/v) and OXC unloaded and loaded L64/F127 1:4 (%w/v) mixed micellar solution. In short, the above mentioned drugs, unloaded and loaded mixed micellar formulations at a concentration range of 200-1000 µg/mL were added to 2 mM solution of $FeCl_2$ (0.05 mL). The addition of a 5 mM solution of ferrozine (0.2 mL) to the abovementioned mixture initiated the reaction and mixture was shaken vigorously for 10 min at room temperature.

2.2.7 Reducing power assay

The method of Oyaizu[35] was adopted to determine the reducing potential of pure drugs CLZ and OXC; empty mixed micelles of L64/P84 1:4 (% w/v) and L64/F127 1:4 (% w/v); CLZ and OXC loaded mixed micellaes of L64/P84 1:4 (% w/v) and L64/F127 1:4 (% w/v) respectively, in a concentration dependent manner. This method is based on the reduction of ferricyanide. A mixture was prepared which contained 1 mL of different concentrations ranging from 200-1000 µg/mL of above mentioned test formulations and 2.5 mL of 0.2 M of sodium phosphate buffer (pH=6.6) containing 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The above reaction mixture was then further incubated at 50°C for 20 min. After incubation, the reaction mixture was acidified by adding 2.5 mL of trichloroacetic acid (10%). The acidified sample (2.5 mL) was then incubated with 2.5 mL of distilled water and 0.1% of $FeCl_3$ (0.5 mL). The absorbance was taken at 700 nm

using UV-visible spectrophotometer. The increase in measured absorbance depicts the greater reduction potential of the reaction mixture.

2.2.8 Nitric oxide (NO) scavenging assay

The method of Garrat *et al.*[36] has been used for the estimation of NO scavenging activity. The reaction mixture containing sodium nitroprusside (10 mM) in a PBS solution, pure drugs CLZ and OXC, CLZ and OXC unloaded and loaded L64/P84 1:4 (%w/v) and L64/F127 1:4 (%w/v) mixed micellar solutions respectively at different concentrations ranging from 200-1000 µg/mL was incubated at room temperature for 2h. On completion of the incubation period, 0.5 mL of Griess reagent was added and the above reaction mixture without the formulation (blank) was taken as control. The absorbance of chromophore was determined at 546 nm.

3. Results and Discussion

3.1 critical micelle concentration (*cmc*) determination

The critical micelle concentration (*cmc*) is a major parameter in determining the *in vitro* and *in vivo* stability of polymeric mixed micelles. The *cmc* values were calculated using fluorescence spectroscopy, as shown in **Table 1**. The findings of the study demonstrated lower *cmc* values for binary mixed micelle system compared to individual systems. It is expected that the PPO and PEO segments of L64 and L35 pluronic tend to interact with both the PPO and PEO segments of higher molecular weight pluronics P84 and F127 that disturb the interactional balance between hydrophobic and hydrophilic segments. This behavior is in accordance with our previous report in which there was a decrease in *cmc* upon interaction of Polyethylene glycol with pluronic L64.[37] This results in the smaller sizes of the mixed micelles as confirmed by DLS measurements. Low *cmc* values of L64/P84 (1:4) and L64/F127 (1:4) mixed micelles demonstrated the high stability of these micelles upon dilution.

Table 1: Critical micelle concentration (*cmc*) values obtained from Fluorescence spectroscopy: Binary mixed micelles of L64 and L35 with different pluronics (P84 and F127) at different % w/v at 37°C.

Systems	**0%+5%**	**1%+4%**	**2%+3%**	**2.5%+ 2.5%**	**3%+2%**	**4%+1%**	**0%+5%**
L64+P84	0.150	0.054	0.049	0.44	0.066	0.070	0.400

L35+P84	0.150	0.063	0.058	0.068	0.088	0.089	0.452
L64+F127	0.035	0.030	0.030	0.034	0.033	0.049	0.400
L35+F127	0.035	0.031	0.039	0.037	0.047	0.062	0.452

3.2 *cmt* determination:

It has been reported that DLS measurements are used to determine the *cmc* or *cmt* values of different surfactant molecules. For *cmt* determination, we have adopted this technique as it is well established fact that due to the aggregation of unimers of surface active molecules as a function of the temperature, there is a rise in the scattered intensity which results to larger particles that indicates the formation of micelles. We have determined the *cmt* values of pluronic L64 mixed with other pluronics *viz.* P84, F68, F127 and P123 is found to be below the drug temperature solubilization of 37^{o}C. Therefore, *cmt* does not affect the solubilization of hydrophobic drugs. So in our study, *cmt* values do not have a direct effect on the solubilization of drugs since solubilization was done at 37^{o}C**.** The *cmt* values of binary system of L64/P84 at concentration ratio of 1:4, 2:3, 2.5:2.5 were found to be at temperature 22^{o}C, but as the concentration of L64 was further increased to 3 and 4% i.e. L64/P84(3:2), L64/P84(4:1) respectively, the *cmt* temperature was also increased (24^{o}C and 26^{o}C respectively). This is attributed to the chemical structure of the L64 which is less hydrophobic than pluronic P84 thus need some extra temperature to form the micelles. A similar trend was observed in the case of binary mixed micelles of L64 with F127 at different % w/v. On the contrary, in the case of mixed micelles of L64/F68 and L64/P123 at different % w/v, a decrease in *cmt* values was observed with increasing concentration. This trend was attributed to the hydrophilic nature of these pluronics. [Showed in Figure 1]

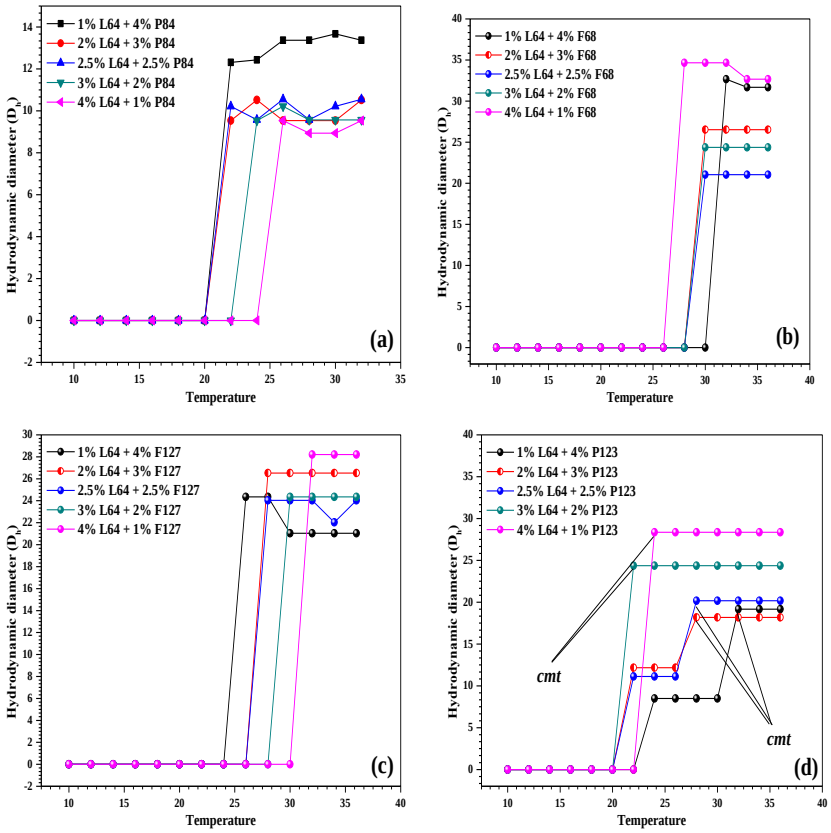


Figure 1: Critical micelle temperature (*cmt*) of binary mixtures of different pluronics at different temperature range **(a)** L64 with pluronic P84, **(b)** L64 with pluronic F68, **(c)** L64 with pluronic F127, and **(d)** L64 with pluronic P123 at various concentrations (%w/v).

3.3 Solubilization of Hydrophobic drugs clozapine (CLZ) and oxcarbazepine (OXC)

The solubilization of CLZ and OXC in aqueous media at room temperature is 0.037 mM and 0.415 mM respectively. To increase their solubility in aqueous media, we have used both pure as well as binary mixtures of lower molecular weight pluronics L64 and L35 and relatively higher molecular weight pluronics P84, P123, F127 and F68 with different % w/v far above their critical micelle concentration (*cmc*) values at 37°C. The solubility of OXC and CLZ in different pure pluronic (P84, F127, F68, P123, L64 and L35) micelles at 37°C was lower than mixed micelles as shown in Table S2 (supplementary information). This may be due to different core sizes providing a hydrophobic pool[38]. Furthermore, it was anticipated that different corona length formed by the lower molecular weight pluronics also allow the drug to solubilize in the core region of the mixed micelles. In Figure 2 (a) and (b), the highest solubilization of CLZ was found in a binary mixture of L64 and L35 (used for solubilization of OXC and CLZ) with high

molecular weight pluronics (P84/F127/F68/P123) at concentration ratio of 1:4 %w/v. On the other hand, the solubilization capacity decreased on decreasing the concentration ratio of high molecular weight pluronics from 4% to 3% whereas the further decrease in concentration ratio of high molecular weight pluronics to 2.5%, the solubilization of drugs was increased. Moreover, when the concentration ratio of high molecular weight pluronics was decreased to 2% and 1% respectively, the solubilization decreased. Among all the binary mixtures, the solubility of CLZ was higher (9.641 ± 0.217 mg/ 5 mL) in the case of a binary mixture of L64/P84(1:4) because of the hydrophobic nature of P84 and L64. Due to the hydrophobic nature of CLZ, the majority of the drug prefers the hydrophobic PPO core region of L64/P84(1:4) mixed micelles.[39] But in the case of OXC, as shown in **Figure 3 (a)** and **(b)**, the highest solubility was observed for a mixture of L64/F127(1:4) (6.712 mg ± 0.223/5mL). This is due to solubilization of OXC both in the PPO core and PEO corona region and attributed to its polar carboxamide moiety (F127 has longer corona region made up of total 130 PEO units).[40-41]

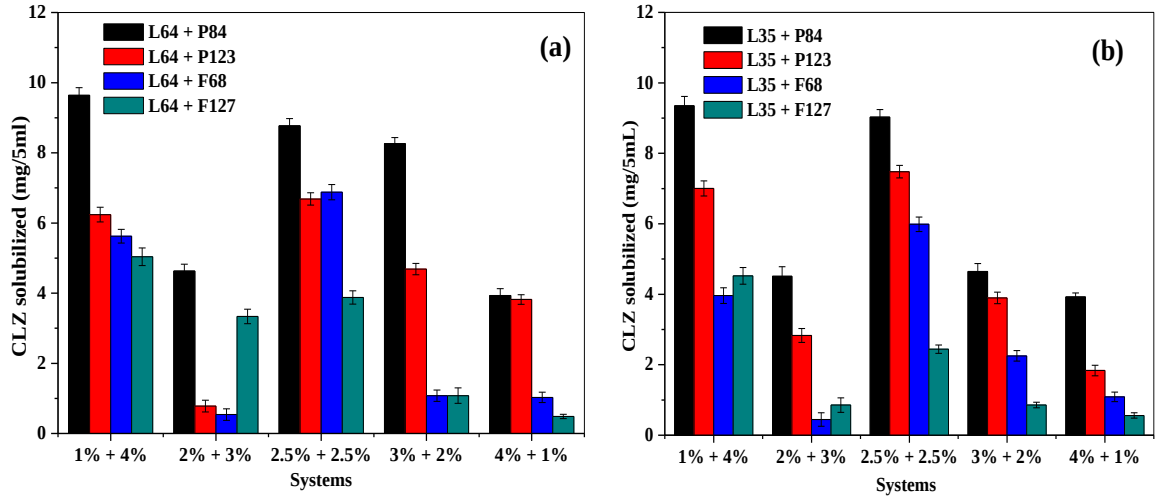


Figure 2 Amount of CLZ solubilized in binary mixtures of **(a)** L64 with high molecular weight pluronics (P84, F127, F68 and P123) **(b)** L35 with high molecular weight pluronics (P84, F127, F68 and P123) at 37°C.

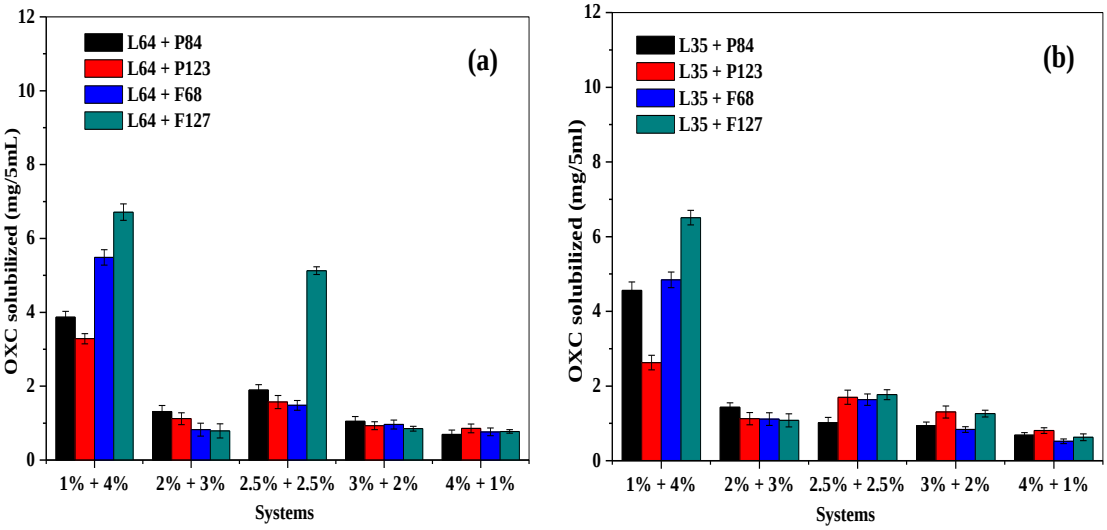


Figure 3 Amount of OXC solubilized in binary mixtures of **(a)** L64 with high molecular weight pluronics (P84, F127, F68 and P123) **(b)** L35 with high molecular weight pluronics (P84, F127, F68 and P123) at 37°C.

The drug loading efficiency is expressed as the percentage of solubilized drug compared to original drug feed in different pluronic micelles as shown in equation (1)

$$Drug\ loading\ efficiency = \frac{weight\ of\ drug\ in\ micelles}{weight\ of\ drug\ fed\ initially} \times 100 \qquad (1)$$

As shown in **Table S3** (supplementary information), the drug loading efficiency of CLZ in the binary mixture L64/P84(1:4) was higher than the other binary mixtures of L64 and L35 with other pluronics (P84, F127, F68 and P123) at all % w/v, indicating the higher drug loading efficiency of this system. In case of OXC, the drug loading efficiency is higher in L64/F127 (1:4) as compared to other binary mixtures of L64 and L35 with other pluronics (P84, F127, F68 and P123) and presented in **Table S3** (supplementary information) respectively.

The micellar/water partition coefficient can be calculated from UV-visible results and is defined as the ratio of drug concentration in the micelle to the drug concentration in water at a particular surfactant concentration (Equation 2).

$$P = \frac{S_{tot} - S_w}{S_w} \qquad (2)$$

where S_{tot} and S_w is the total solubility of the drug and solubility of drug in water respectively. Among all the binary mixtures of L64 and L35 with other pluronics (P84, F127, F68 and P123), the highest value of the partition coefficient for CLZ was found in case of L64/P84(1:4). The partition coefficient values for OXC was found to be higher in a system comprised of a mixture of L64/F127 (1:4) amongst all the binary mixtures

due to solubilization of OXC in both the core and corona regions. These values are represented in Table S4 of the supplementary information.

The standard free energy of solubilization (ΔG^o) can be determined from the partition coefficient and is given by the following expression (3).

$$\Delta G^\circ = -RTlnP \qquad (3)$$

where R is the gas constant, T is the temperature (in Kelvin), and P is the partition coefficient between the micelle and the aqueous phase. The ΔG^o values depict the Gibbs free energy of transfer of one mole of the drug in the micellar phase. The ΔG^o values were found to be negative, indicating the spontaneity of the solubilization and transfer of water molecules from the micellar core persuading a more favourable environment and hence leading to spontaneous partition of OXC into the micelles.[42-45]

As shown in **Table 2,** in the case of binary mixtures of high molecular weight pluronics (P84/F127/F68/P123) with L64 and L35 (used for solubilization of OXC and CLZ) at concentration ratio of 1:4, the value of ΔG^o was observed to be highest. On the contrary, the value of ΔG^o becomes less negative on decreasing the concentration ratio of high molecular weight pluronics from 4% to 3%, the further decrease in concentration ratio of high molecular weight pluronics to 2.5% led to the more negative value of ΔG^o. Moreover, when the high molecular weight pluronics concentration ratio was further decreased to 2% and 1%, less negative ΔG^o values were obtained which show less spontaneity of drug solubilization. Higher negative values of ΔG^o in case of CLZ were encountered in mixtures of L64/P84(1:4) amongst all other binary systems, which is attributed to the more hydrophobic nature of this binary mixture. On the contrary, in case of OXC solubilization, the highest negative value of ΔG^o was observed in L64/F127(1:4) micellar system indicated a spontaneous OXC solubilization process which can be ascribed to the solubilization of polar OXC in both the core and corona region of this binary system.

Table 2: Standard free energy of (CLZ and OXC) solubilization (*ΔG°)* in the mixed micelles of L64 and L35 with high molecular weight pluronics (P84, F127, F68 and P123) at 37°C.

Binary	**CLZ**			
System	**L64+P84**	**L64+F127**	**L64+F68**	**L64+P123**
1%+4%	-14.768 ± 0.060	-13.436 ± 0.138	-13.741± 0.095	-14.028 ± 0.092
2%+3%	-12.806 ± 0.110	-12.294± 0.170	-7.056 ± 0.905	-8.175 ± 0.620
2.5%+2.5%	-14.515 ± 0.062	-12.714 ± 0.135	-14.298 ± 0.087	-14.219 ± 0.072
3%+2%	-14.356 ± 0.055	-9.104 ± 0.581	-9.115 ± 0.430	-13.239 ± 0.095
4%+1%	-12.367 ± 0.131	-6.798 ± 0.383	-8.982 ± 0.408	-12.671 ± 0.097
	L35+P84	**L35+F127**	**L35+F68**	**L35+P123**
1%+4%	-14.685 ± 0.0759	-13.138 ± 0.145	-12.772 ± 0.156	-14.425 ± 0.081
2%+3%	-12.733 ± 0.159	-8.430 ± 0.694	-6.409 ± 1.331	-11.836 ± 0.194
2.5%+2.5%	-14.593 ±0.062	-11.427 ± 0.135	-13.914 ± 0.093	-14.529 ± 0.065
3%+2%	-12.813 ± 0.130	-8.468 ± 0.261	-11.201 ± 0.184	-12.727 ± 0.115
4%+1%	-12.361 ± 0.077	-7.223 ± 0.403	-9.147 ± 0.348	-10.630 ± 0.226
Binary	**OXC**			
System	**L64+P84**	**L64+F127**	**L64+F68**	**L64+P123**
1%+4%	-5.633 ± 3.983	-7.460 ± 0.097	-6.867 ± 0.113	-5.303 ± 0.132
2%+3%	-2.034 ±1.438	0.287 ± 0.155	0.174 ± 1.224	-1.421 ± 0.632
2.5%+2.5%	-3.374 ± 2.386	-6.665 ± 0.060	-2.580 ± 0.342	-2.794 ± 0.417
3%+2%	-1.094 ± 0.773	-0.114 ± 0.402	-0.724 ± 0.607	-0.573 ± 0.566
4%+1%	1.160 ± 0.820	0.411 ± 0.260	0.538 ± 0.835	-0.127 ± 0.753
	L35+P84	**L35+F127**	**L35+F68**	**L35+P123**
1%+4%	-6.121 ± 0.143	-7.369 ±0.087	-6.494 ± 0.129	-4.587 ± 0.242
2%+3%	-2.370 ± 0.315	-1.252 ±0.735	-1.396 ± 0.676	-1.436 ± 0.634
2.5%+2.5%	-0.950 ± 0.643	-3.237 ±0.269	-2.947± 0.344	-3.085 ± 0.406
3%+2%	-0.602 ± 0.493	-1.949 ±0.298	-0.009± .506	-2.070 ± 0.499
4%+1%	1.160 ± 0.628	1.988 ±1.215	3.942 ± 1.678	0.156 ± 0.039

3.4 Dynamic light scattering (DLS) measurements

DLS is a versatile technique for the determination of size changes in colloidal particles.[46] The size of drug carrier is also important in drug delivery because it plays an important role in drug accumulation and penetration in cells i.e. the micelle size should be large

enough for circulation as well as small enough for their penetration.[47] Among all the binary mixtures of pluronics formulations, L64 and L35 with P84 show higher solubilization for CLZ. In case of OXC, binary mixture of L64/F127 (1:4) showed higher solubilization. Therefore, DLS measurements of these selected mixed micellar formulations were observed to further explore their potential as drug carriers.[38] To understand the effect of drug solubilization on the mixed micellar size of the pluronics, DLS studies were performed with loaded and empty binary mixed micellar solutions. The D_h value of pure P84 was 13.54 ± 0.1 nm. The D_h value [from **Figure 3 (a-e**)] of empty binary mixed micelles of L64 with P84 at different % w/v *viz.* L64/P84(1:4), L64/P84(2:3), L64/P84(2.5:2.5), L64/P84(3:2), L64/P84(4:1) were found to be 10.971, 8.391, 9.574, 9.553 and 8.949 ± 0.1 nm respectively. Pluronic L64 has only 26 PEO (corona forming hydrophilic portion) and 30 PPO units (core forming hydrophobic portion) which is smaller than pluronic P84 that has 38 units PEO and 40 PPO units. The concentration of P84 was when decreased and concentration of L64 increased, the hydrodynamic diameter (D_h) of mixed micelles decreased due to shorter length of core and corona segments of pluronic L64. It is well established that DLS technique measures the D_h and it will be reflected if any changes occur in the corona region or the solvent associated with it. [37, 48] On the other hand, after solubilization of CLZ in the binary mixtures of pluronics, D_h of CLZ loaded mixed micelles were found to be 16.615, 13.757, 15.056, 18.235 and 14.802 ± 0.1 nm, respectively [**Figure 4 (a-e**)]. A significant difference was observed between the D_h of unloaded and loaded binary mixed micelles of different pluronics, indicating that CLZ was solubilized in the core of mixed micelles of the pluronics and was responsible for swelling of the mixed micelles. In the binary mixture of L64/F127(1:4),the size of empty mixed micelle was 21.045 ± 0.1 nm and size of OXC loaded mixed micelle was 24.880 ± 0.1 nm as shown in **Figure 4 (f)**. There was a significant increase in the size of the mixed micelles because OXC solubilized both in PPO core and PEO corona region. In the binary mixtures of L35 with P84 at different % w/v, D_h (shown in **Figure S1**) of empty binary mixed micelles were found to be 13.80, 14.122, 14.468, 14.802 and 16.420 ± 0.1 nm in L35/P84(1:4), L35/P84(2:3), L35/P84(2.5:2.5), L35/P84 (3:2) and L35/P84 (4:1) respectively. On the other hand, D_h of CLZ loaded binary mixed micelles L35/P84 (1:4), L35/ P84(2:3), L35/P84(2.5:2.5), L35/P84 (3:2) andL35/P84 (4:1) were found to be 16.919, 18.747, 19.810, 20.128 and

20.371 ± 0.1 nm. These results also showed that there was swelling of mixed micelles after the addition of CLZ which confirm its solubility in different binary mixed micelles.

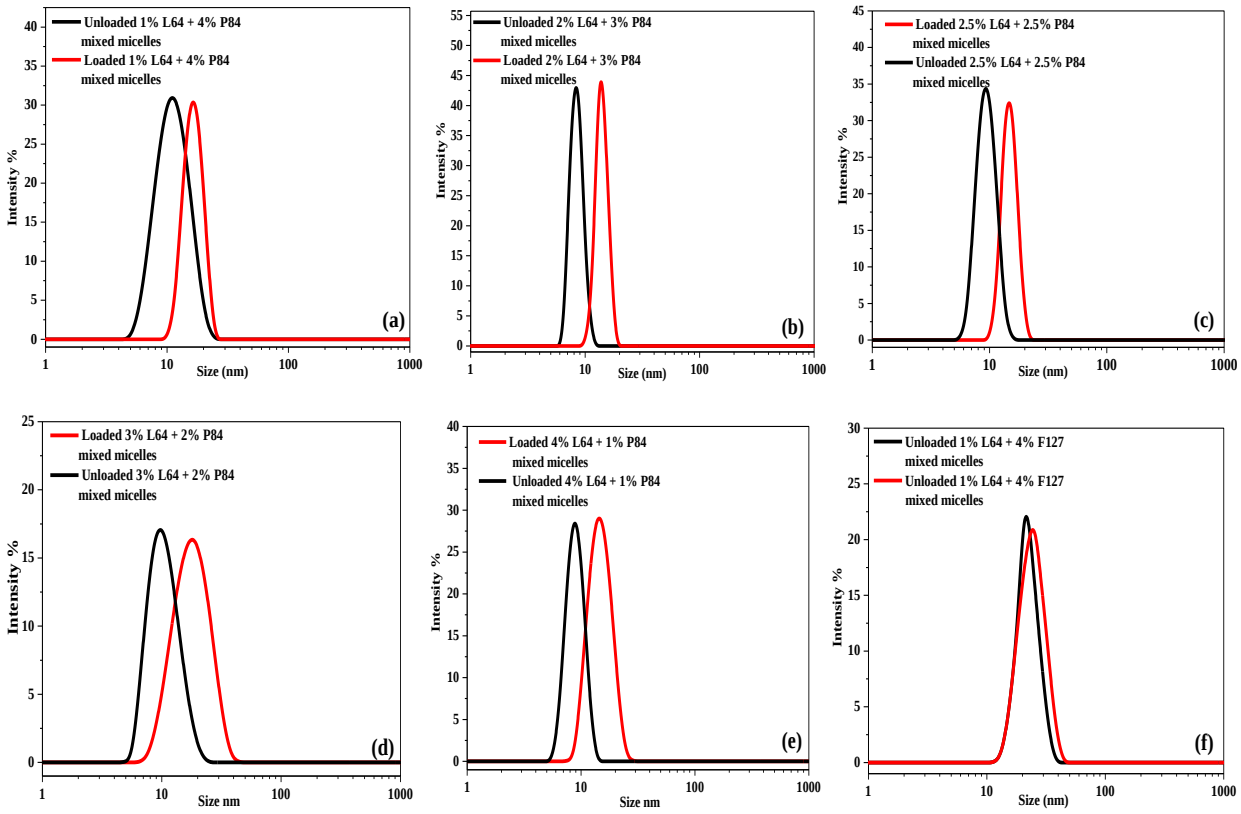


Figure 4: Intensity weighed size distribution profiles for empty and CLZ loaded mixed micelles of L64 + P84 at different % w/v **(a)** 1% w/v L64 + 4% w/v P84 **(b)** 2% w/v L64 + 3% w/v P84 **(c)** 2.5% w/v L64 + 2.5% w/v P84 **(d)** 3% w/v L64 + 2% w/v P84 **(e)** 4% w/v L64 + 1% w/v P84; **(f)** OXC loaded mixed micelles of 1% w/v L64 + 4% w/v F127.

3.5 *In vitro* drug release

In vitro drug release from the micellar medium is an important test since it provides an idea about their behaviour *in vivo*.[19] To determine the release of CLZ loaded in the binary mixture of L64 and L35 with P84 and OXC release in the binary mixture of L64/F127(1:4) the dialysis bag method has been employed. In the binary mixed micelles of L64 with P84, CLZ release profile shows that there was 65.56%, 78.02%, 76.84%, 72.66% and 100% release from the binary mixtures of L64/P84(1:4), L64/P84(2:3), L64/P84(2.5:2.5), L64/P84(3:2), L64/P84(4:1) respectively after 5h [**Figure 5 (a)**]. The CLZ drug release becomes slow after initial burst release. 100% drug release from the binary mixtures of L64/P84(1:4), L64/P84(2:3), L64/P84(2.5:2.5) and L64/P84(3:2)

takes 26 h, 22 h, 20 h and 20 h respectively. This shows that the release become slower with increased amount of P84.

On the other hand, in the binary mixtures of L35 with P84, burst CLZ release was observed from the binary micelles *viz.* L35/P84(1:4), L35/P84(2:3), L35/P84(2.5:2.5), L35/P84(3:2) and L35/P84(4:1) *i.e.* 58.53%, 61.37%, 85.44%, 73.03% and 100% respectively in 5 h [**Figure 5 (b)**]. The release slowed down with time and it takes 56 h, 48 h, 22 h and 28 h to release from the binary mixtures L35/P84(1:4), L35/P84(2:3), L35/P84(2.5:2.5) and L35/P84 (3:2) respectively. This shows a similar trend as the above mentioned binary systems (L64/P84) and indicates that with the increase in amount of P84, the release slowed down.

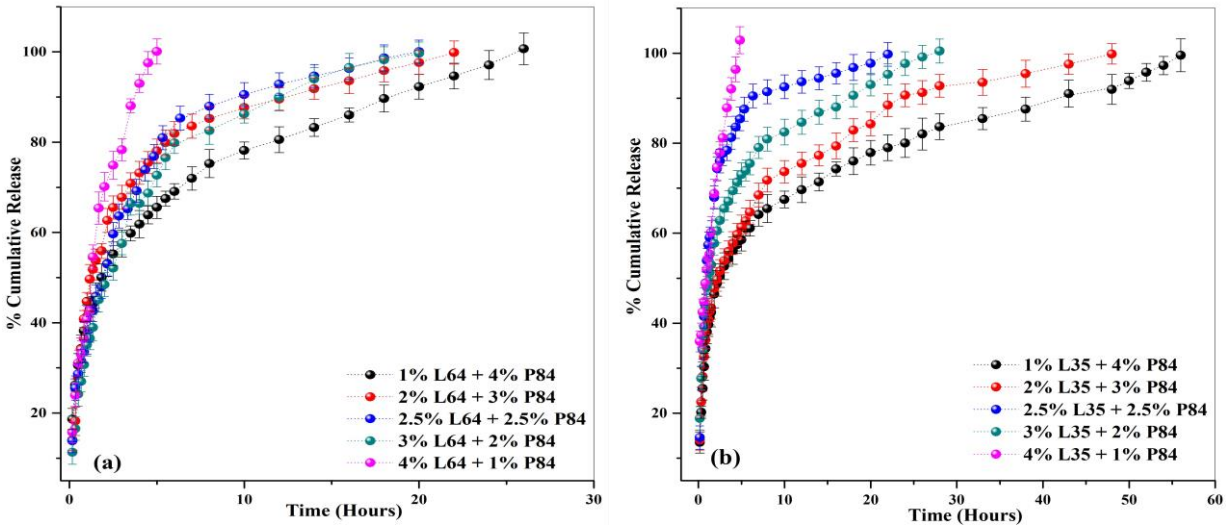


Figure 5: *In vitro* release of CLZ from binary mixtures of **(a)** L64 with P84 at different % w/v **(b)** L35 with P84 at different % w/v.

In case of OXC, it showed a sustained release from the binary system of L64/F127(1:4) but release was faster than pluronic P84 formulations. This was attributed to the longer hydrophilic corona region of pluronic F127 that results in faster release of OXC. After 5 h, 74% of the drug was released (shown in **Figure S2**). The 100% drug was released after the 20 hours.

The drug release data was analysed using five mathematical models *viz*. zero-order kinetics, first-order kinetics, Higuchi kinetics, Hixson-Crowell model and the Korsmeyer-Peppas (KP) model. Correlation coefficient (R^2) and rate constant (K) values were calculated for all five models and presented in **Table S5** and **Table S6.** The most appropriate model was chosen based on the R^2 values of the fit. In the binary mixtures of

L64/P84(1:4), L64/P84(3:2), L64/P84(4:1), L35/P84(1:4) and L35/P84(2:3) the R^2 values obtained from Korsmeyer-Peppas model are higher than the other models. The corresponding value of N for binary mixtures *viz*. L64/P84(1:4), L64/P84(3:2), L35/P84(1:4) and L35/P84(2:3) was less than 0.45 which means these formulation follow the Fickian diffusion mechanism, whereas in mixtures of L64/P84(4:1) the value of N was greater than 0.45 which corresponds to non-Fickian transport.[49]

On the other hand in the binary systems of L64/P84(2.5:2.5), L35/P84(2.5:2.5) and L35/P84(3:2), higher values of R^2 were obtained by employing the first order kinetics model that describes systems where the drug release rate is independent of the concentration of the dissolved drug. In the binary mixture of L64/P84(2:3), the highest value of R^2 was obtained for the Hixson-Crowell model which shows that the release is from the systems where there is change in diameter and surface area of particles. In the binary mixture of L35/P84(4:1) the highest value of R^2 was obtained for the Higuchi model kinetics suggesting that CLZ release is diffusion controlled. In the binary system of L64/F127(1:4) containing OXC, the highest value of R^2 was also attributed to the first order kinetics model.[50]

3.6 Antioxidant assays

3.6.1 DPPH radical scavenging activity

The DPPH radical scavenging activity (%) was determined for pure drugs CLZ and OXC, CLZ and OXC unloaded and loaded mixed micellar formulation of L64/P84(1:4) and L64/F127(1:4) respectively, depicted in **Figure 6 (a)**.[51] After addition of both the test formulations and free drugs at a concentration ranging from 200-1000 µg/mL, partial free radicals consumption was observed. Our results evidenced that higher radical scavenging activity of CLZ loaded mixed micellar formulation of L64/P84(1:4) and found to be 79.88 % at a concentration of 1000µg/mL was found compared to unloaded mixed micelles of L64/P84(1:4) and the free drug (CLZ). In case of OXC loaded mixed micelles of L64/F127(1:4), the % DPPH scavenging activity was found to be 49% at a concentration of 1000 µg/mL which indicated a lower radical scavenging activity compared to L64/P84(1:4)/CLZ mixed micelles [**Figure 6 (a)**]. Moreover IC_{50} (50 % inhibitory concentration) of CLZ loaded mixed micelles was also calculated and was found to be 481.4 µg/mL as shown in **Figure S3 (a)** in the supplementary information**.**

There was a significant statistical difference between the mean DPPH radical scavenging values ($p < 0.01$). The higher antioxidant activity of CLZ loaded mixed micelles of L64/P84(1:4) can be attributed to the increased particle distribution and smaller size of L64/P84(1:4) micelles.[52]

3.6.2 Metal chelating activity

The method by Dinis *et al.* was used to estimate the chelating of ferrous ions by pure drugs CLZ and OXC, CLZ and OXC unloaded and loaded mixed micellar formulation of L64/P84(1:4) and L64/F127(1:4) respectively.[34] As shown in **Figure 6 (b),** both CLZ and OXC loaded mixed micellar formulation of L64/P84(1:4) and L64/F127(1:4) respectively interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. There was a linear decrease in the absorbance of the ferrozine-Fe^{2+} complex in a concentration dependent manner (from 200 to 1000 µg/mL). The higher metal chelating activity of CLZ loaded mixed micellar formulation of L64/P84(1:4) was observed compared to unloaded mixed micellar formulation and CLZ alone. At concentration of 1000 µg/mL, the percent metal chelating capacity of CLZ unloaded and loaded mixed micellar formulation of L64/P84(1:4) was found to be 20% and 78% respectively. Although OXC loaded mixed micelles L64/F127(1:4) also showed good metal chelating activity as compared to OXC alone. The percent metal scavenging capacity (at 1000 µg/mL) of OXC unloaded and loaded L64/F127(1:4) mixed micellar formulation was found to be 10% and 52% respectively. IC_{50} value of CLZ loaded mixed micelles of L64:P84 was found to be 343.2µg/mL [**Figure S3 (b)**] in the supplementary information, whereas OXC loaded L64:F127 mixed micelles showed IC_{50} at 755.3µg/mL [**Figure S3 (c)** in the supplementary information]**.** From IC_{50} values it can be evidenced that CLZ loaded mixed micellar formulation has better antioxidant activity as compared to OXC loaded mixed micellar formulation.[53]

3.6.3 Reducing Power assay

The reducing power method of Oyaizu was applied to quantify the antioxidant activity of pure drugs CLZ and OXC, CLZ and OXC unloaded and loaded mixed micellar formulation of L64/P84(1:4) and L64/F127(1:4) respectively.[35] As can be seen from **Figure 6 (c)**, both the mixed micellar formulations at different concentrations (200-1000 µg/mL) showed effective reducing power activity when compared to pure drugs. The reducing power of investigated formulations and free drugs increased steadily as with

increase in concentration. The reducing power of both mixed micellar formulations and drug alone at higher concentration (1000 µg/mL) were in the following order: L64/P84(1:4)/CLZ > L64/F127(1:4)/OXC > L64/P84(1:4) >CLZ >L64/F127(1:4) > OXC. The results demonstrated that both formulations neutralized free radicals by forming stable products and can be attributed to electron donating capacity of investigated formulations.[54-56]

3.6.4 Nitric Oxide (NO) scavenging activity

The method of Garrat *et al.* was employed to study the NO scavenging activity of pure drugs CLZ and OXC, CLZ and OXC unloaded and loaded mixed micellar formulation of L64/P84(1:4) and L64/F127(1:4) respectively.[31] **Figure 6 (d)** represents the comparative NO scavenging activity of the both mixed micellar formulations and drugs alone. Both mixed micellar formulations exhibited NO scavenging by reducing nitrite concentration in the assay medium in concentration dependent manner (200-1000 µg/mL). The mixed micellar formulations of CLZ loaded L64/P84(1:4) and OXC loaded L64/F127(1:4) showed maximum activity of 78.06% and 58.48% (at 1000 µg/mL) respectively as compared to drug alone whereas NO scavenging activity for unloaded mixed micelles of L64/P84(1:4) and L64/F127(1:4) was found to 30% and 20% respectively. IC_{50} value of CLZ and OXC loaded mixed micellar formulations was found to be 383.9µg/mL and 756.9µg/mL respectively as shown in **Figure S3 (d)** and **(e)** respectively. A decrease in the absorbance at 550 nm by the test compound is linked to the NO scavenging capacity. [57]

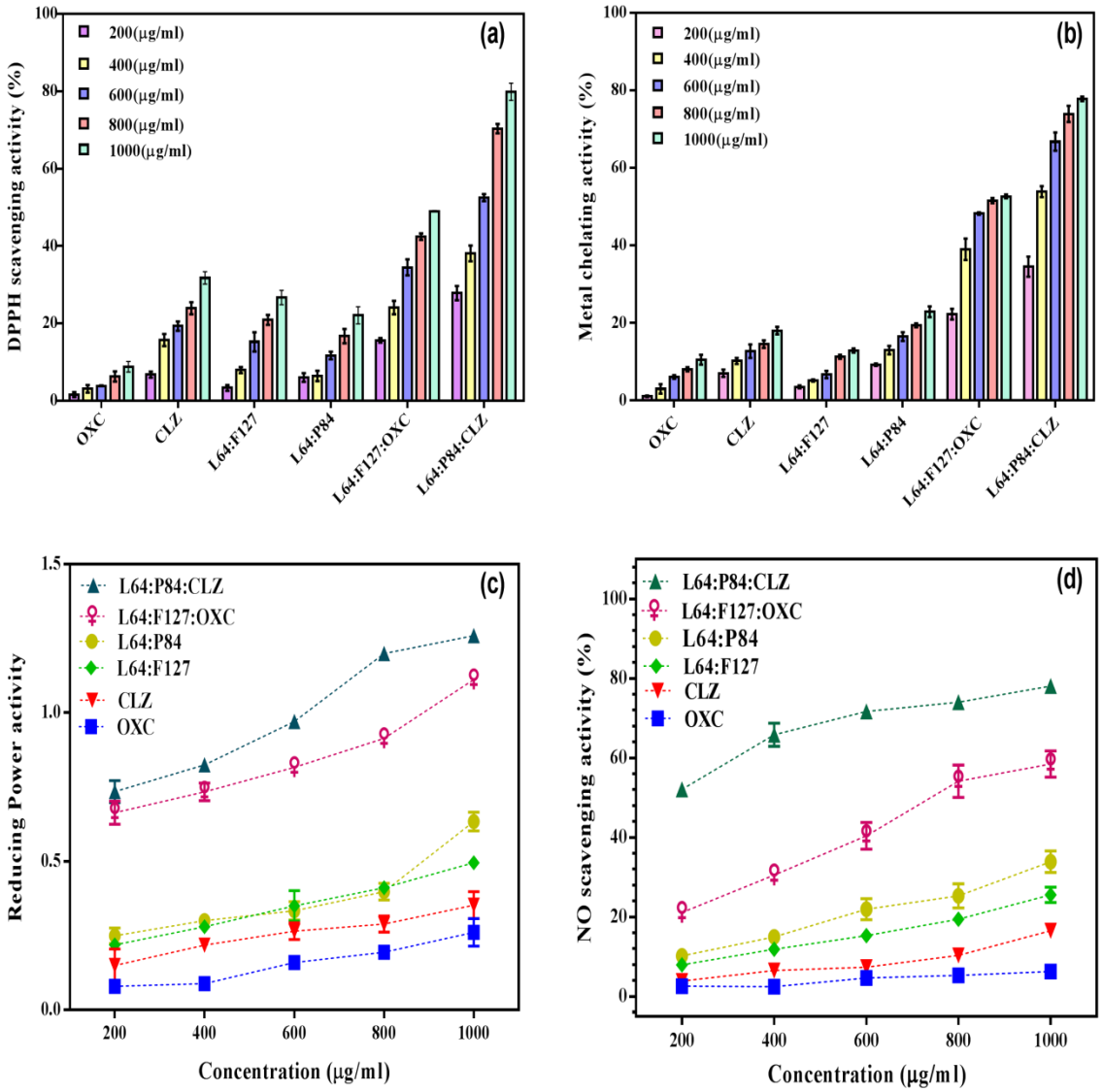


Figure 6: The antioxidant activity **(a)** DPPH scavenging activity (%) **(b)** Metal chelating activity (%) **(c)** Reducing power activity **(d)** NO scavenging activity (%) of bare drugs (CLZ and OXC), OXC and CLZ unloaded and loaded mixed micellar formulation of L64:F127(1:4) and L64:P84(1:4) respectively.

4 Conclusions

In this work, we studied the solubilization of the hydrophobic drugs clozapine (CLZ) and oxcarbazepine (OXC) in binary mixed micelles of low molecular weight pluronics L64 and L35 and high molecular weight pluronics P84, F127, F68 and P123. From the obtained results, it was concluded that the highest solubility of CLZ was observed in binary mixture of L64/P84(1:4) due to the hydrophobic nature of P84 and L64. In case

of OXC, the highest solubility was observed in L64/F127 (1:4) because the drug solubilized both in the core and the corona region of F127 since OXC contains a carboxamide polar moiety. From dynamic light scattering (DLS) measurements, it was concluded that there was an increase in the size of mixed micelles from empty mixed micelles to drug (CLZ and OXC) loaded mixed micelles. Furthermore, fluorescence spectroscopy indicated that the *cmc* decreased with the formation of mixed micelles as compared to pure individual micelles. *In vitro* release study of CLZ showed that as the amount of P84 in the binary mixtures of L64/P84 and L35/P84 decreases, the release become faster. Therefore, it is possible to control the drug release from the pluronic micelles by simply varying the concentration of one pluronic. The results of DPPH scavenging activity assay, Metal chelating activity assay, Nitric oxide and Reducing power assays evidenced significantly higher antioxidant activity of the mixed micellar formulations compared to the unloaded drugs (CLZ and OXC). It is anticipated that the formulations L64/P84(1:4)/CLZ and L64/F127(1:4)/OXC may act as the efficient drug carriers for the drug delivery of CLZ and OXC respectively.

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