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Fabrication of Oxaliplatin Microemulsion for Enhanced Cytotoxicity against Colon Cancer Cells

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Abstract— *The aim of this study was to explore the potential of microemulsion (ME) to improve oxaliplatin efficacy against colon cancer cells. Oxaliplatin ME was prepared by water titration method using Miglyol 812 as oil phase, tween 80 and labrasol as surfactant and cosurfactant respectively. The constructed phase diagram showed the positioning of the ME towards surfactant/cosurfactant region. All the prepared ME formulae with negatively charged with particle size less than 100 nm. The conductivity results revealed that the ME could be water in oil or oil in water. Water amount could significantly affect conductivity, particle size and viscosity of the proposed ME. The incorporation of oxaliplatin in water in oil ME controlled the in vitro release for 24 h in contrast to only 2 h for oxaliplatin oil in water ME. The imparted lipophilicity produced by ME, especially water in oil ME, improved cytotoxicity against CT26 colon cancer cells by 1.7-2 fold. These results gave a rationale for further in vivo biological and toxicological studies.*

Keywords— *Controlled drug release, Cytotoxicity, Lipophilicity, Microemulsion, Oxaliplatin.*

I. INTRODUCTION

Microemulsions (ME) are colloidal carriers that have attracted interest due to their potential in overcoming some of other nanocarriers limitations as instability and drug leakage [1]. ME are thermodynamically stable, isotropically clear dispersion of two immiscible liquids stabilized by an interfacial film of surfactant molecules [2]. They offer a great penetration enhancement effect due to their surfactants and cosurfactants content with a small droplet size of 10-100 nm [3]. The existence of microdomains of different polarity within the same single-phase solution enables both hydrophilic and lipophilic materials to be solubilized [4].

Depending on the proportion of suitable components and hydrophilic–lipophilic balance (HLB) value of the surfactants used, the formation of microdroplets can be in the form of oil-swollen micelles dispersed in the aqueous phase as for the o/w ME or water-swollen micelles dispersed in oil as the w/o ME. In the intermediate phase region between o/w and w/o ME, there may exist bicontinuous ME where aqueous and oil domains are interconnected randomly in the form of sponge-like microstructures [5].

Beside its ability to incorporate both hydrophilic and lipophilic drugs, this system has been reported to protect the incorporated drugs against oxidation, enzymatic degradation and enhance the membrane permeability [6]. This enhanced penetration activity is mainly due to an increase in drug concentration and thermodynamic activity which provides a large concentration gradient from the vehicle to the site of application [7]. The small droplets also provide better adherence to membranes and transport drug molecules in a controlled manner, in addition to ME contents of surfactant and cosurfactant. Other advantages include higher physical stability in plasma than liposomes or other vesicles and ease of sterilization by filtration [8].

The choice of the ME type is usually based on the application of the system. The protection of water soluble drug molecules, in particular proteins and peptides from metabolism, overcoming physical barriers and controlling the release of hydrophilic drugs are some of the important reasons for exploring w/o type. Higher bioavailabilities have been found in animals after administration of vasopressin and insulin formulated in w/o ME [9]. Moreover, targeting the lymphatic system, through which the peritoneal fluid is mainly absorbed, could be better achieved using lipid-based systems [10]**.** In case of cancer, this is very important since the tumor cells disseminated to the peritoneal cavity specifically infiltrate the milky spots in the greater omentum leading to ascites development. Growth of the tumor in lymphoid tissue might induce further accumulation of malignant ascites [11]**.** Preparation of w/o ME is thought to be promising to deliver hydrophilic compounds to lymph systems which could be an appropriate strategy to avoid metastasis accompanying colon cancer.

Assuming that a model anticancer hydrophilic drug, oxaliplatin, will distribute mainly in the inner aqueous phase of a w/o ME. Herein, it was hypothesized that loading oxaliplatin in w/o-ME, is thought to control oxaliplatin release and improve cytotoxicity. Therefore, oxaliplatin ME was prepared in different types either w/o or o/w and the release as well as the cytotoxicity of oxaliplatin will be evaluated along with other characterization and stability tests.

II. MATERIALS AND METHODS

Materials

Oxaliplatin, Tween 80, RPMI, fetal bovine serum (FBS), penicillin, streptomycin was purchased from Sigma–Aldrich Company, St. Louis USA. Potassium dihydrogen ortho phosphate, sodium hydroxide, sodium chloride, potassium chloride, sodium dibasic hydrogen ortho phosphate and hydrochloric acid was supplied from Fluka Chemika-

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BioChemika, Switzerland. Acetonitrile (HPLC grade) was purchased from Riedel-de Haen Gmbh, Germany. Labrasol was a kind gift from Gattefossé, France. Miglyol 812 was supplied from Caesar & Loretz GmbH, Germany.

Methods

Phase Diagram Construction

For the preparation of ME, Miglyol 812 was used as oily phase, tween 80 as surfactant and labrasol as cosurfactant. The surfactant and co-surfactant, in 1:1 weight ratio, were vortex mixed vigorously for 30 sec forming the surfactant mixture (Sm). Oil was added to Sm in different ratios ranging from 9:1 to 1:9 w/w in screw capped vials and the mixture was vortex mixed to ensure thorough mixing. Oxaliplatin (4% w/w) was dissolved in the oil/Sm by vortex mixing. Different drug oil/Sm ratio was diluted drop wise with normal saline, vortex mixed for 30 seconds and the obtained mixture was subsequently stored at room temperature [12]. The ME domains were identified by visual inspection for clarity and fluidity.

In vitro Microemulsion Characterization:

Conductivity Measurement:

The electric conductivity values for different oxaliplatin ME were measured by dipping the electrode of a portable digital conductometer (Hanna, Hungaria) in a suitable amount of 20 g of each of ME maintained in a water bath at 37 °C. The recorded readings were those stable for 20 min.

The dynamic electric conductivity of oxaliplatin loaded ME was determined at different temperatures in the range of 5^{o}C to 50^{o}C, in 1^{o}C increment. Phase inversion temperature (PIT) was calculated as the average of the temperatures of minimum and maximum conductivity values [13].

Determination of Droplet Size and Zeta Potential:

The droplet size and size distribution expressed as polydispersity index (PDI) as well as zeta potential of oxaliplatin ME formulae were measured by dynamic light-scattering technique (DLS) using the Zeta sizer (Malvern Instruments Ltd., UK) at an angle of 90°.

Viscosity Determination

The viscosity of 5 mL sample volume of each of selected oxaliplatin formulae was evaluated at 37^{o}C using Brookfield DV-III (USA) ultra-programmable cone and plate rheometer, fitted with a spindle number 40. Brookfield Rheocalc operating software was controlling the rheometer.

Transmission Electron Microscopy (TEM) of Oxaliplatin Microemulsion

The selected oxaliplatin microemulsion formulae were visualized using (TEM). A drop of each of the selected formula was deposited on a copper 300-mesh grid, coated with carbon, and allowed to stand for 10 min after which any excess fluid was absorbed in a filter paper. Before examination, one drop of 1% phosphotungstic acid was applied and allowed to dry for 5 min.

In vitro Release of Oxaliplatin from Selected Microemulsion

In vitro release of oxaliplatin from ME formula was conducted by the dialysis method [14]. Briefly, 1 g of the prepared oxaliplatin ME (4% w/w) was placed in dialysis membrane (cut-off 1000 Da). The dialysis membranes were placed into 200 mL PBS pH 7.4 at 37°C in stoppered conical flask which was placed on a thermostatically controlled shaker rotating at 50 ± 1 rpm. At predetermined time intervals, aliquots of 1mL of the release medium were sampled and the same amount of fresh dissolution medium was added into the container to maintain a constant volume. The samples were assayed using HPLC system (Agilent 1100, Germany) equipped with G 1311A quaternary pump and UV detector (VWD-G1314 A). A reverse phase C18 column (Thermo® BDS, 150X4.6 mm, 5µ) was used at 25^{o}C. The wavelength of the UV detector was set at 255 nm. Mixture of phosphoric acid (0.01 M) and acetonitrile (95:5 v/v) was used as mobile phase at a flow rate of 1 mL/min [15].

Physical Stability of Selected Formulae

Thermal Stability:

The selected oxaliplatin ME formulae were kept at 40 ±2^{o}C and RH 75± 5% for 6 months according to accelerated ICH Q1A stability protocol. Change in color, phase separations and turbidity were observed by naked eye. Particle size measurements and zeta potential of the aforementioned ME were also conducted as previously discussed.

Gravitational Stability:

Oxaliplatin ME formulae were subjected to accelerated physical stability testing in which the chosen formulae were centrifuged at 3750 rpm at 25º C for 5 hours and the formulae were tested for phase separation and turbidity if any.

In vitro Cytotoxicity:

RPMI media with FBS (10% v/v), L-glutamine (1%), penicillin (50 U/mL) and streptomycin (50 µg/mL) was used to culture CT26 colon cancer cells. After 80% confluency, cells were seeded at density 7K cells/ well for 24 h in 96-well plate. The cells were incubated with oxaliplatin solution and ME serial concentration of oxaliplatin in the range 0.01-100 µM. Plain ME was also tested in the same concentration range to check the possible toxicity of ME. After 72 h incubation period, media was aspirated and MTT solution (120 µl) was added on the cells for 4 h at 37°C and 5% CO_2. Then DMSO (200 µl) was added to dissolve the formed formazan crystals and the absorbance was measured at 570 nm by plate reader (FLUO star OPTIMA, BMG Labtech).

Statistical analysis

All experiments were done in triplicate. Data are presented as mean of the three replicates for each experiment ± SD. Unpaired student-t test was used for comparing between two variables and probability values *P* value <0.05 was considered significant.

III. Results and Discussion

Phase Diagram Construction

The pseudo-ternary phase diagram of Miglyol 812, tween 80: labrasol in a weight ratio of 1:1 and normal saline is displayed in Fig. 1. It is obvious that the ME region was positioned towards surfactant/cosurfactant rich zone. The use of sodium chloride did not adversely affect the system [14]. It has been previously reported that electrolytes increase the interdroplet interactions and viscosity of the continuous phase [16] and enhance the non-ionic surfactant lipophilicity by salting out effect [17].

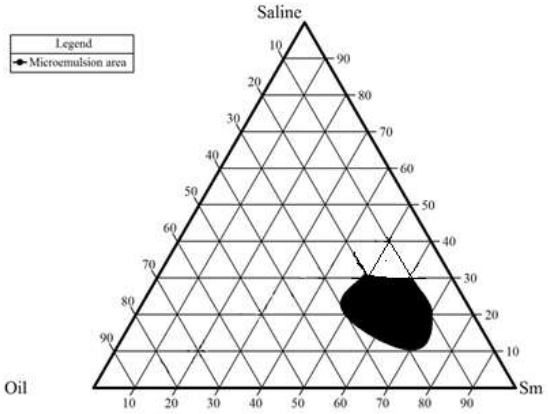

Fig. 1. Phase Diagram of oxaliplatin microemulsion Composed of Miglyol 812 (Oil), normal saline (aqueous phase) and mixture of tween 80 with labrasol (in 1:1 ratio).

Characterization of Oxaliplatin Microemulsion:

Conductivity Measurement:

Table 1 shows the composition and characteristics of the prepared ME formulae based the phase diagram. All the formulae possessed low conductivity values (<1 µS/cm) and could therefore be considered as w/o ME except formulae 11 and 12 [18].

Generally, when water molecules are dispersed in an oil phase, water molecules are disconnected from each other and exhibits minimum interactions and liquid conductivity is low. On the contrary, o/w ME with higher water content the total number of aqueous droplets which can increase the formation transient clusters (aggregation of water molecules) to increase liquid conductivity [19]. Therefore, a significant increase in conductivity was also observed in o/w formulae ($P<0.05$).

Determination of Droplet Size and Zeta Potential

Table 1 represents the particle size and PDI of the tested oxaliplatin ME formulae. Increasing water to surfactant ratio leads to a linear decrease in ME particle size. Similar results were previously reported where particle size decreased with water to surfactant ratio increase with a narrower PDI [14]. PDI is strictly below 0.2 favoring a monodispersed system formation [20]. Small particle size allows the sterilized through a sterile syringe-driven filter avoiding thus the thermal treatment [21].

Current chemotherapy of cancer is still facing a major problem of lack of selectivity of anticancer drugs toward tumor cells, thus cells of the bone marrow and gastrointestinal tract which are rapidly proliferating are getting affected by the cytotoxic action of these drugs. This results in a narrow therapeutic index of most anticancer drugs. Along with this, increasing resistant types of tumors require high dose of anticancer drugs which in turn enhances the toxicity of treatment [22]. The mechanisms of nanoparticle-cell interaction are still not completely understood. A previous study indicated that the internalization of nanoparticles was size dependent [23]. Endocytosis of particles with size less than 100 nm plays a vital role in cancer treatment [24].

Based on these facts ME formulae F6 (w/o) or F12 (o/w) and was chosen for further studies. These formulae have particle size lye in the desired range for endocytosis.

Negative zeta potential values (table 1), found with all ME formulae, were possibly imparted by the free fatty acids present in the oil phase and/or surfactants [25]. A decrease in zeta potential was noted by increasing normal saline content probably due to the decrease in thickness of electrical double layer [26].

Dynamic Conductivity:

Fig. 2 shows the dynamic conductivity of F6 and F12. Generally the behavior of spontaneous curvature of non-ionic surfactant monolayer based system could be tailored by temperature [27]. Phase inversion temperature (PIT) can be determined by monitoring the changes of the conductivity values with temperatures in non-ionic surfactant system [28].

As shown in Fig. 2, F6 conductivity was found to be (≈2.6 µS/cm) and did not show any significant difference in the range 5°C to 24°C where the first maximum (1st max) was seen with a value of 2.88 µS/cm ±0.18 at 25°C. By increasing the temperature, the conductivity dropped to 2.34 µS/cm ± 0.11 (1st min) at 26°C before it increased again forming the 2nd max at 29°C (2.73 µS/cm ±0.24). The conductivity suddenly dropped to 0.017 µS/cm ± 0.002 (2nd min) at 34°C. Hence, PIT temperature was calculated and was found to be 29.5^{o}C.

On the contrary, the dynamic conductivity of F12 was found to be almost constant in the temperature range of 5^{o}C to 33^{o}C (3.62 µS/cm ±0.04 to 3.65 µS/cm ±0.07 respectively) then an increase in conductivity with first maximum (1st max) at 34^{o}C with conductivity value of 4.59 µS/cm ± 0.06 was observed. Further increase in temperature showed decrease in conductivity value reaching 3.29 µS/cm ±0.12 at 35^{o}C (1st min) followed by increase forming the 2nd max at 38^{o}C (3.71 µS/cm ±0.11). Finally the conductivity values suddenly dropped to 0.041 µS/cm ± 0.008 at 45^{o}C. Therefore, PIT temperature was calculated and was found to be 40^{o}C.

The high conductivity at temperature lower than 25^{o}C and 38^{o}C for formula F6 and F12 respectively was due to the formation of o/w ME, while the respective zero conductivities found at temperature higher than 30^{o}C and 40 ^{o}C for F6 and F12 respectively indicated the formation of a w/o system. Therefore the prepared systems is expected to be w/o for F6 and o/w for F12 at physiological temperature. These results could be due to the surfactant molecules tendency to assemble in a way to minimize the bending energy between oil and aqueous phases. At low temperature, the spontaneous

curvature (H_0) was positive and micelles were formed in water. The oil was solubilized by the micelles forming Winsor I system which describes the equilibrium between o/w ME (aqueous micellar solution) and excess oil.

By increasing the temperature, the affinity of non-ionic surfactant monolayer spontaneous curvature to oil increased due to the dehydration of surfactant hydrophilic portion forming probably Winsor II equilibrium (w/o ME in equilibrium with excess aqueous phase) dominated [27].

So it can postulate that F6 at low temperature the ME was of the o/w type. This would be beneficial in terms of ease of dilution during the preparation at room temperature. As the temperature increased, inversion occurred at 29.5°C and such inversion was considered to be favorable in terms of sustaining drug release, one of the study targets.

Viscosity Measurement

The respective measured viscosity values for F6 and F12 were 33.25 ±3.54 cP and 42.95 ±1.25 cP. It is obvious that formula F12 showed significantly higher viscosity than F6 ($P<0.05$). In ME, the viscosity values tended to increase slightly when the water concentrations increased. The hydrophilic chains of surfactant, in presence of water, are expected to be strongly hydrated and connected with hydrogen bonds allowing the interaction between the droplets causing viscosity increase [13]. It is obvious that the viscosity of both formulae was less than 120cP indicating suitability for needle injections [29].

TABLE 1. Composition of oxaliplatin microemulsion, corresponding conductivity values, particle size and zeta potential:

Formula	O (%w/w)	Sm (%w/w)	Saline (%w/w)	Conductivity (µS/cm)±SD	Type	Particle size measurements ±SD		Zeta Potential (mV) ±SD
						Mean (nm)	PDI	
F1	32	58	10	0.014 ±0.004	w/o	65.66±2.08	0.101±0.200	-18.80±0.30
F2	30	60	10	0.015±0.004	w/o	67.33±0.57	0.112±0.009	-19.40±1.04
F3	27	59	14	0.020±0.003	w/o	58.66±3.51	0.099±0.001	-17.70±0.55
F4	26	58	16	0.023±0.002	w/o	47.33±2.51	0.076±0.022	-17.33±0.66
F5	24	55	21	0.029±0.004	w/o	26.66±1.52	0.026±0.005	-15.23±0.40
F6	23	50	27	0.026±0.01	w/o	22.33±1.54	0.014±0.004	-12.80±0.20
F7	21	56	23	0.038±0.006	w/o	26.33±1.52	0.025±0.002	-13.50±0.36
F8	18	54	28	0.042±0.008	w/o	23.33±1.52	0.017±0.002	-11.26±0.68
F9	16	67	17	0.027±0.002	w/o	51.33±3.51	0.096±0.018	-15.80±0.55
F10	15	57	28	0.044±0.005	w/o	24.33±1.15	0.019±0.002	-11.63±0.25
F11	15	55	30	1.54±0.14	o/w	22.13±0.96	0.025±0.012	-10.18±0.87
F12	10	60	30	2.60±0.20	o/w	20.50±0.95	0.01±0.002	-8.38±1.00

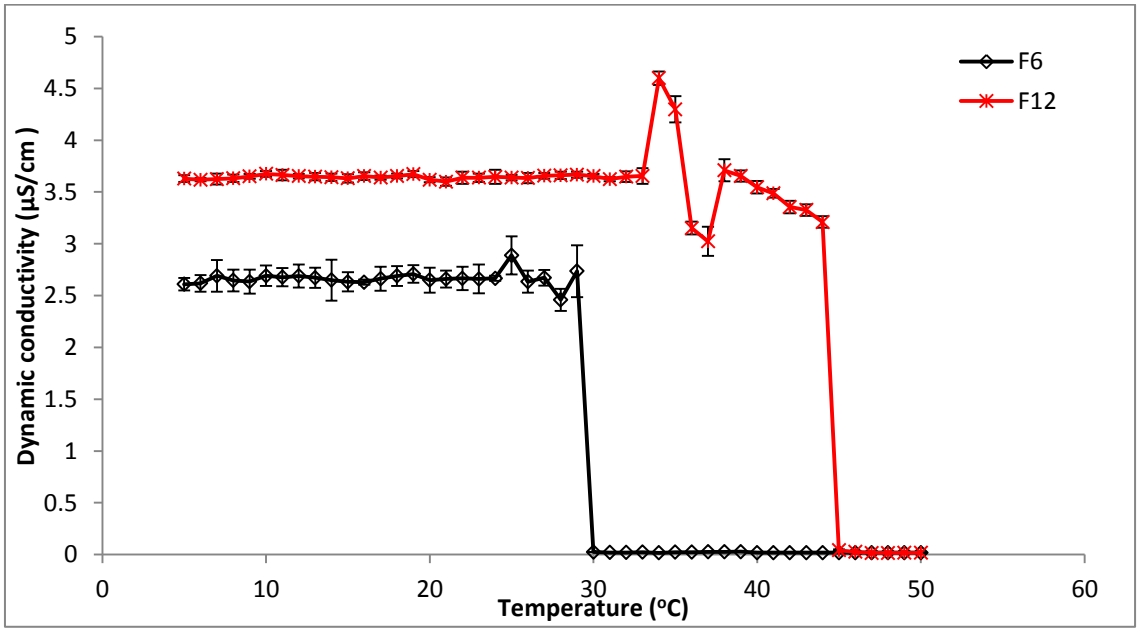

Fig. 2. Dynamic conductivity values of formula F6 and F12 as function to different temperatures.

Transmission Electron Microscopy (TEM) of Oxaliplatin Microemulsion

Fig (3A and B), reveals that oxaliplatin loaded ME globules of F6 and F12 were spherical non-aggregated with diameters range 20-30 nm which is in good agreement with the results of particle size determined by DLS.

In vitro Release of Oxaliplatin from Selected Microemulsion:

In vitro release of oxaliplatin from ME formulae F6 and F12 was studied using dialysis method. The corresponding release profiles are illustrated in Fig 4. $T_{100\%}$ was almost reached after 24 h and 2 h for formulae F6 and F12 respectively. Oxaliplatin is a hydrophilic drug with a partition coefficient log $P_{(octanol/water)}$ of -0.47 [30]. Accordingly

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oxaliplatin possesses a notable affinity for aqueous phase and favored residence in it. Hence, in formula F6 (w/o), oxaliplatin needed to diffuse from the inner aqueous phase to the outer oily layer which acted as a barrier sustaining drug release for 24 h. On the contrary, in formula F12 (o/w), oxaliplatin might have resided mainly in the external aqueous phase, in direct contact with the dissolution medium causing rapid drug release. In fact, w/o ME have been considered efficient as prolonged release systems for hydrophilic drugs with the oil and emulsifier layers acting as release barriers [31].

Physical Stability of Selected Formulae

Neither change in clarity nor phase separation were observed at any of the tested formulae after centrifugation proving the stability of all selected formulae against gravitational effect even under stress conditions. Both formulae kept their original color with no phase separation or turbidity with no significant difference (P>0.05) between particle size, PDI or zeta potential of all selected formulae when comparing the result obtained before and after storage (table 2).

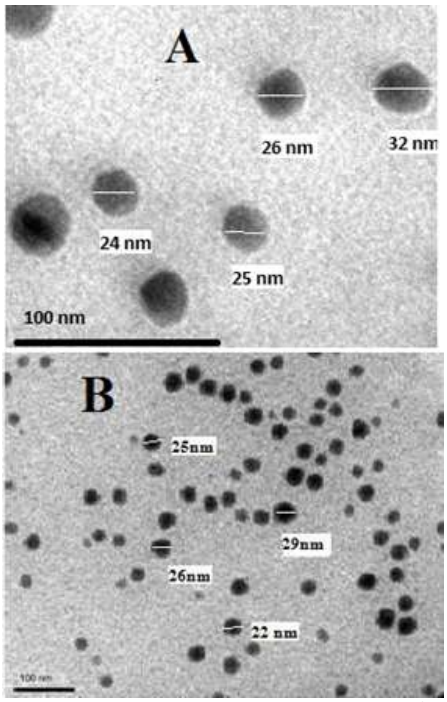

Fig. 3. Transmission electron microphotography (TEM) of oxaliplatin Microemulsion F6 (A) and F12 (B).

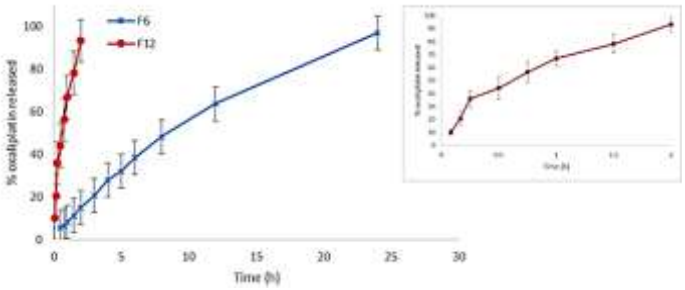

Fig. 4. In vitro release of oxaliplatin from microemulsion F6 and F12 Formulae in PBS (pH 7.4)

In vitro Oxaliplatin Cytotoxicity Assessment:

The cytotoxicity of oxaliplatin ME (F6 and F12) and solution as well as the corresponding plain formulae was evaluated on CT26 cancer cells using MTT assay. Fig 5 illustrates the relation between % cell viability after treatment with the different formulae for 72 h and oxaliplatin concentration. It is obvious that there was a rapid decline in viability of cells treated with solution, F6 and F12 reflecting the strong cytotoxic effect of oxaliplatin in concentration dependent manner.

The IC_{50} values was found to be 0.1749 µM, 0.216 µM and 0.375 µM for oxaliplatin F6, F12 and solution respectively. The cytotoxicity of oxaliplatin F6 and F12 is ≈1.7-2 fold that of oxaliplatin solution. High cell viabilities exceeding 70% were obtained with both blank formulae (plain F6 and F12) up to a concentration equivalent to 200-300 fold the IC_{50}. No significant difference (*P>0.05*) in cell viability was found between the two plain formulae suggesting the absence of any substantial effect for the used excipients. The slight decrease in cell viability seen with both plain formulae could be attributed to the presence of surfactants.

Previous studies demonstrated that the degree of cytotoxicity is correlated with the amount of platinum bound to DNA which depends on its concentration and exposure time with tumor cells [32]. As we used same exposure time for all formulae, the increased antitumor activity of both oxaliplatin F6 and F12 over oxaliplatin solution could be attributed to the increased lipophilicity of the former systems imparted by the oily phase of the ME leading to a probable increase in drug penetration into the tumor cells [31]. Furthermore, by virtue of its permeability enhancement activity, the used surfactants might have played a role in the noticed cytotoxicity [33]. Another possible mechanism is that ME absorption can take place via the cellular uptake of the drug through an endocytotic pathway of droplets or by droplet fusion with the cell membrane, leading to increased internalization of the droplets and drug release inside the tumor [34]**.** The determined particle size of both w/o and o/w ME globules was lying in the typical optimum size range for endocytosis [35], supports this hypothesis.

The noticed improved cytotoxicity of oxaliplatin F6 (w/o) over F12 (o/w) ME could be attributed to the shielding the drug in w/o ME might help protecting it from attack and degradation by Glutathione. A deactivated conjugates of oxaliplatin have been readily excreted by a Glutathione - conjugated export pump [36].

Regardless the ME type, it is therefore proposed that the ME is a potentially suitable carrier system for the anticancer drug oxaliplatin for therapeutic purposes and may be useful in intraperitoneal administration for carcinomatosis. Among the other expected advantages from such system is the possibility to deliver the hydrophilic drug to the lymphatic system owing to the presence of the oil. As the peritoneal fluid is mainly absorbed via the lymphoid tissue and the tumor growth in lymphoid tissue might induce further accumulation of malignant ascites. This strategy is thought to avoid metastasis accompanying colon cancer [11].

TABLE 2. Effect of storage different oxaliplatin microemulsion at 40 ±2°C and RH 75± 5% for 6 months on particle size, PDI and zeta potential

Parameter	F6		F12	
	Freshly prepared	After 6 months	Freshly prepared	After 6 months
Particle size (nm)± SD	22.33±1.54	24.32±2.24	20.50±0.95	22.35±1.97
PDI ± SD	0.014±0.004	0.015±0.003	0.01±0.002	0.014± 0.003
Zeta potential (mV) ± SD	-12.80±0.20	-13.12±0.11	-8.38±1.00	-8.1±0.54

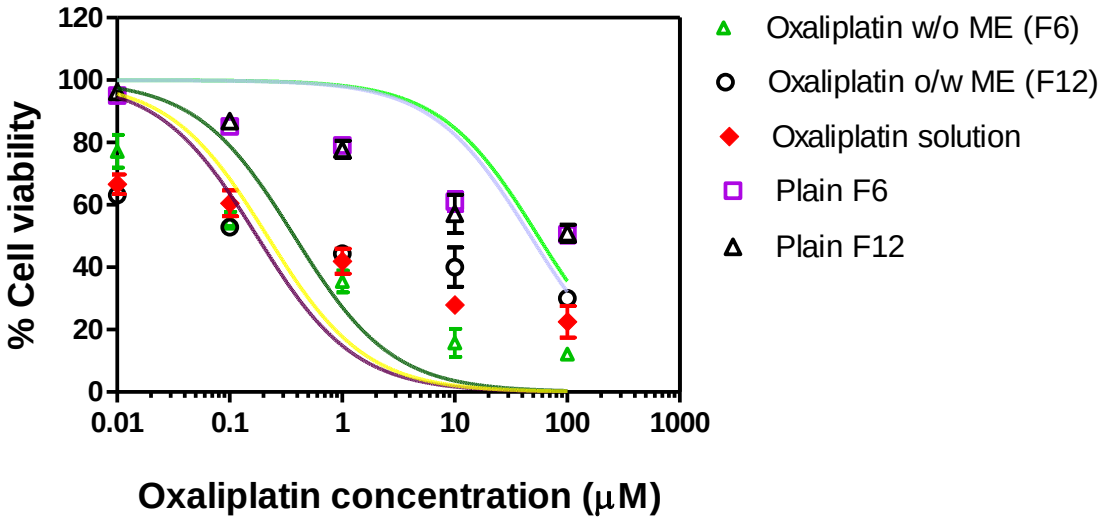

Fig. 5. Cell viability after 72 h exposure at 37°C to different concentrations of oxaliplatin in solution and w/o microemulsion (F6) and o/w (F12), plain F6 and F12. Results are mean of three determinations ± standard deviation (SD).

IV. Conclusion

From the above results, oxaliplatin ME was successfully prepared using Miglyol 812, tween 80 and labrasol. The obtained particle size was less than 100 nm with negative zeta potential. The type of ME could be determined using dynamic conductivity measurements and was found to be dependent on the temperature. Moreover, it could be deduced that loading oxaliplatin into w/o ME could provide an efficient way to control drug release for 24 h. The cytotoxicity of oxaliplatin ME either w/o or o/w was ≈1.7-2 fold more than oxaliplatin solution. The promising improved cytotoxicity of ME would give a rational for further studies.

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