RESEARCH ARTICLE

Formulation and *In-vitro* Evaluation of Methotrexate Nanoemulsion using Natural Oil

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ABSTRACT

Objective: The objective of the research was the preparation and evaluation of methotrexate nanoemulsion to improve its solubility, permeability, and bioavailability.

Methods: The formulation components were selected based on the solubility study, and the pseudo-ternary phase diagrams were constructed by the aqueous phase titration method.

Result and discussion: The prepared nanoemulsions were exposed to several thermodynamic stability studies and then to many characterizations tests for the selection of the best formulation. F 11 is the best formula since it has a 57 nm globule size, 0.21 polydispersity index, +23.67 zeta potential, and 99.57% light transmittance. FTIR study confirmed no incompatibility between drug and excipients.

Conclusion: F 11 was a promising nanoemulsion formula that improved methotrexate permeability and bioavailability.

Keywords: Bioavailability, Methotrexate, Nanoemulsion, Solubility.

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INTRODUCTION

Nanoemulsion is a transparent heterogeneous system made up of two immiscible liquids (water and oil) that are stabilized by an interfacial layer of surfactant/co-surfactant mixture (S-mix), to produce an isotropic system that contains drug molecules within oil phase droplets.[1]

The generated nanoemulsion dispersion's droplets have a homogeneous distribution with droplet sizes ranging from 20 to 200 nm, allowing for high drug fluxes and penetration while allowing the nano-sized droplets to penetrate readily.[2]

Four types of nanoemulsion present: nanoemulsion of oil in water (o/w), with the oil phase disseminated in the continuous aqueous phase, nanoemulsion of water in oil (w/o), comprised of an internal water phase scattered within a continuous oil phase, a bi-continuous nanoemulsion in which the oil and water phases are disseminated throughout the system at the same time and (w/o/w) or (o/w/o) multiple nanoemulsions.[3]

Nanoemulsion is composed of four ingredients, including oil, water, surfactants, and co-surfactants.

The oil assists in the solubilization of the selected lipophilic drug candidate. The oil is chosen based on its highest drug solubility to guarantee maximal drug loading in the nanoemulsions. Oils used in nanoemulsion could be natural, synthetic or semi-synthetic oils.

Surfactant must be able to solubilize the oil phase with high affinity and strong solubilizing ability to the lipophilic drug molecules, which is critical for nanoemulsion generation. Surfactant is either nonionic, zwitterionic, cationic, or anionic.[4]

Co-surfactant is a short-chain amphiphilic molecule that helps surfactant molecules to decrease the oil-water interfacial tension to an ultralow negative or near-zero value, which is necessary for nanoemulsion generation. Furthermore, co-surfactants increase the miscibility of the hydrophilic surfactant and the drug with the oil phase, resulting in increased spontaneous emulsification and formulation stability. The most often utilized co-surfactants include intermediate chain alcohols such as hexanol and octanol and other organic solvents such as propylene glycol, ethanol, and polyethylene glycol.[5]

Methotrexate (MTX) a folic acid antagonist, has been utilized to treat a variety of malignancies as well as autoimmune and inflammatory illnesses. MTX is poorly soluble in water (less than 1mg/ml) with low permeability (log P -1.8) (BCS type IV class). MTX has pKa values of 4.8 and 5.5, bioavailability

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60% at lower doses, less at higher doses, protein binding 35–50% (parent drug), 91–93% (7-hydroxymethotrexate), metabolized by hepatic and intracellular metabolism.[5] Elimination half-life is 3–10 hours (lower doses), 8–15 hours (higher doses), and MTX average molecular weight of 454.4 Da.[6]

MTX usage is restricted due to its limited water solubility, permeability, oral bioavailability, short half-life, and significant side effects.

Hepatotoxicity, nephrotoxicity, neurotoxicity, and pulmonary toxicity are the most common adverse effects of MTX.[7]

Improvement of the drug delivery mechanism through formulation development has been necessary to boost therapeutic effects while overcoming these limitations.

Micelles, microspheres, nanoparticles, and liposomes are some of the MTX formulations that have been developed to improve MTX bioavailability.[6,7]

As a result, the goal of this study is to develop and characterize a MTX (o/w) nanoemulsion to improve MTX solubility as well as permeability resulting in enhanced total bioavailability.

MATERIALS AND METHODS

Materials

MTX was bought from YIBAI biotechnology (China), Triton X-100, Triton X-114, Cremophor, Span 80, Tween 80, and Tween 20 were purchased from Sigma ch. (USA), Anise, Almond, Argan, Olive, Castor, Triacetin, and Eucalyptus oil were provided from HIMEDIA chemicals India, Peppermint, Cod Liver and Cumin oil were purchased from BAR-SUR-LOUP (France), Dimethyl sulfoxide (DMSO), Ethanol, Iso Amyl Alcohol, Propylene Glycol, Transcutol P, PEG 400, PEG 200, Labrasol and Carbitol were bought from Merck (Germany). Franz cell was purchased from SES GmbH (Germany).

Methods

Determination of MTX Solubility

MTX solubility was measured in a variety of oils, including (anise, almond, argan, olive, castor, triacetin, eucalyptus, peppermint, cod liver, and cumin oil) as well as surfactants (Triton X-100, Triton X-114, cremophor, span 80, tween 80 and tween 20) and co-surfactants (DMSO, methanol, ethanol, iso amyl alcohol, propylene glycol, transcutol p, PEG 400, PEG 200, labrasol and carbitol).

Excess amount of MTX was mixed by Vortex mixer with 2 mL of oils, surfactants, and co-surfactants in a tiny plain glass tube.

The tube was firmly closed and shaken for 72 hours at 25 +/- 0.05°C on an isothermal shaker water bath, after which the specimens were centrifuged for 10 minutes at 6000 rpm. After appropriate DMSO dilution, the solution was filtered, and solubility was evaluated using a UV spectrophotometer at the λ max 302 nm.[8,9] The tests were carried out in triplicate.

Construction of Pseudo Ternary Phase Diagram

To detect the nanoemulsion region and determine the component ratios for nanoemulsion formulation, a pseudo ternary phase diagram of oil, S-mix, and deionized water was developed using the aqueous titration method.[10] The weight ratios of surfactant/co-surfactant of S-mix screened for nanoemulsion formation were 1:1, 1:2, 1:3, 1:4, 2:1, 3:1, and 4:1. These ratios were used for a detailed study of phase diagram which reflects increasing concentrations of co-surfactant with respect to surfactant and increasing concentrations of surfactant concerning co-surfactant.[11] For each phase diagram, different weight ratios of oil and S-mix combined in a range from 1:9 to 9:1 in separated vials, where a homogenous mixture of oil and S-mix was formed. The prepared combinations were then titrated slowly with an aqueous phase of deionized water in a drip-wise manner at room temperature under continuous stirring and visual observation for transparency or clarity.[12] The ternary Plot generator program was used to create the pseudo ternary phase diagram. The colored area of the plot represents the nanoemulsion area, and the broader region showed stronger nano-emulsifying activity.

Formulation of MTX-loaded Nanoemulsion

The selected dose of MTX was added to the required amount mixture (previously mixed and heated to 45◦C) Eucalyptus oil, tween 20, and DMSO with continuous stirring. After cooling to room temperature, with gentle mixing, the aqueous phase was gradually titrated, drop by drop, until an isotropic transparent nanoemulsion was formed, with a dose of 0.6 mg MTX per 1-g of nanoemulsion.

A 14 MTX-loaded nanoemulsion formulation was prepared, as shown in Table 1.

Thermodynamic Stability Studies of MTX Nanoemulsion Formulation

Thermodynamic tests include:

Table 1: Composition (w/w %) of MTX nanoemulsion formulation

Formula name	*S mix ratio*	*Eucalyptus oil %*	*S mix % (Tween 20 + DMSO)*	*Water %*
F 1	1:1	5	35	60
F 2	2:1	5	35	60
F 3	3:1	5	35	60
F 4	4:1	5	35	60
F 5	1:2	5	35	60
F 6	1:3	5	35	60
F 7	1:4	5	35	60
F 8	1:1	5	30	65
F 9	2:1	5	30	65
F 10	3:1	5	30	65
F 11	4:1	5	30	65
F 12	1:2	5	30	65
F 13	1:3	5	30	65
F 14	1:4	5	30	65

Centrifugation Test

The MTX nanoemulsion formulas were centrifuged for 30 minutes at 4000 rpm, then visually examined for phase separation, creaming, or cracking. The next thermodynamic test would be performed on the formulations that passed this test.[13] The measurements were performed in triplicate.

Heating – Cooling Test

This experiment was carried out to see how temperature changes affected the stability of MTX nanoemulsions that had been created.

The produced nanoemulsions were subjected to six heating-cooling cycles, with each formulation being stored at 0 and 45°C for at least 48 hours at each temperature. Cracking, precipitation, or phase separation, and their impact on the stability of nanoemulsion formulations, were then detected in the samples.[14]

Freezing – Thawing Test

This testing was performed for rapid stability assaying of MTX nanoemulsion formulations. The formulations were tested at two different temperatures (-21°C and 21°C) for a total of 24 hours for each temperature test. The tested formulation were observed for phase separation.[15]

Characterization of the prepared MTX nanoemulsion

Droplet Size Measurement

The average particle size of MTX o/w nanoemulsion droplets was measured by Malvern particle size analyzer.

To avoid multiple scattering, the tested samples were prepared by diluting a small amount of nanoemulsion in distilled water and then introducing it to the device for measurement.[16]

Polydispersity Index (PDI) Measurement

Malvern particle size analyzer was used to determine PDI, which measures droplet size homogeneity in nanoemulsion. PDI ranges from 0 to 1.

The lower the uniformity of globule size of nanoemulsion, the higher the polydispersity value.[17]

Zeta Potential Measurement

Malvern zeta sizer was used to assess the nanoemulsion's zeta potential.

The findings were recorded when the samples were put in clean disposable zeta cells.

Cuvettes were washed with methanol before each test and then put the sample to be measured.[18]

pH Measurement

The pH of the produced MTX nanoemulsions was determined using a digital pH meter that had been calibrated with standard buffer solutions before use.

The pH measurement is necessary to ensure that the manufactured nanoemulsion is compatible with the pH of the application site to avoid irritation.[19] The experiment was carried out in triplicate.

Dilution Test

The dilution test was performed to assess the stability of produced MTX o/w nanoemulsion formulations and also to determine their type (o/w or w/o).

Each formulation was diluted to 1:10 and 1: 100 volume ratios with distilled water and then visually inspected for turbidity or phase separation.

The creation of a transparent solution after dilution of a nanoemulsion indicates that it is an o/w nanoemulsion.[20]

Electrical Conductivity Measurement

Using a digital conductivity meter, the electroconductivity of each MTX o/w nanoemulsion formulation was evaluated to validate the type of the created nanoemulsion.

The metal electrodes were dipped in 5 mL of each sample and read at room temperature.[21] The experiment was carried out in triplicate.

Transmittance Percent (T %) Measurement

The optical transparency of the obtained MTX o/w nanoemulsion was assessed using a UV-visible spectrophotometer. The sample was scanned for light transmittance from 200 to 700 nm, and the result at 650 nm was recorded, the tested samples were not diluted, and distilled water was used as a control.[22] The experiment was carried out in triplicate.

Dye Solubilization Test

By mixing 2 mg/ml of methyl orange solution (water-soluble dye) with each formulation and visually observing whether the dye spread evenly (o/w nanoemulsion) or not (w/o nanoemulsion) through a continuous phase, this test was used to determine the nature of continuous phase for each prepared MTX nanoemulsion.[23]

Drug Content Measurement

A UV-visible spectrophotometer was used to determine the drug content.

Using DMSO as a solvent, the absorbance was measured at 302 nm. The tests were carried out in triplicate.

The drug content was calculated using the equation below:
Drug content = (Analyzed content/Theoretical content) * 100

***In-vitro* Drug Release Study**

The *in-vitro* release of MTX nanoemulsion formulations was studied utilizing a vertical Franz cell diffusional system with a receptor portion volume of 40 mL and a dialysis membrane (Mw 8000-14000 D, USA) as a diffusional barrier or membrane. Phosphate buffer was used as releasing media at 37 ± 1◦C under continuous stirring.

A total of 1-g of the prepared MTX nanoemulsion and control, which contain the dispersion of pure MTX in phosphate buffer, were placed separately in the Franz diffusional cell donor compartment.

A total of 1-mL samples were withdrawn from the receptor compartment every 5 minutes. And replaced with the same volume of fresh phosphate buffer to maintain sink condition.[24] Drug content of the samples was quantified by UV- visible spectroscopy at 299 nm. The samples were assayed in triplicate.

Drug – Excipients Compatibility Study

Fourier transformed infrared spectroscopy (FTIR) was used to investigate interactions between MTX and excipients.

The IR spectra of pure MTX and the physical mixture of MTX with all components of nanoemulsion at a ratio (1:1) was measured with the range chosen from 500 cm^{-1} to 4000 cm^{-1}.[25]

Statistical Analysis

Analysis of variance (ANOVA) was used for data analysis in this research, using ($p \leq 0.05$) for the significance. The results were studied in an average of triplicate (n = 3).

RESULTS AND DISCUSSION

Determination of MTX Solubility

The results of MTX solubility in nanoemulsion components showed that the solubility in ten different oils was in the following decreasing order; Eucalyptus > Peppermint > Cumin > Anise > Argan > Cod Liver > Olive > Castor > Almond > Triacetin oil as shown in Table 2.

The solubility of MTX in surfactants was in the following descending direction; Tween 20 > Cremophor > Tween 80 > Span 80 > Triton x-114 > Triton x-100, as presented in Table 2.

The solubility of MTX in co-surfactants was in the following descending path; DMSO > Transcutol P > Carbitol > Labrasol > PEG 200 > Propylene glycol > PEG400 > Ethanol > Capryol > Iso amyl alcohol, as shown in Table 2.

Accordingly, the nanoemulsion constituents were Eucalyptus oil as oil, tween 20 as a surfactant, and DMSO as a co-surfactant was chosen for the production of the nanoemulsion system based on solubility investigations.

Construction of Pseudo Ternary Phase Diagram

The aqueous titration method was used; the oil phase was eucalyptus oil, tween 20 was the surfactant, and DMSO was the co-surfactant.

Seven phase diagram were plotted separately at each S-mix ratio of 1:1, 1:2, 1:3, 1:4, 2:1, 3:1, 4:1. The colored area represents nanoemulsion area, and the wide colored area means better nano-emulsifying activity, as shown in Figure 1.

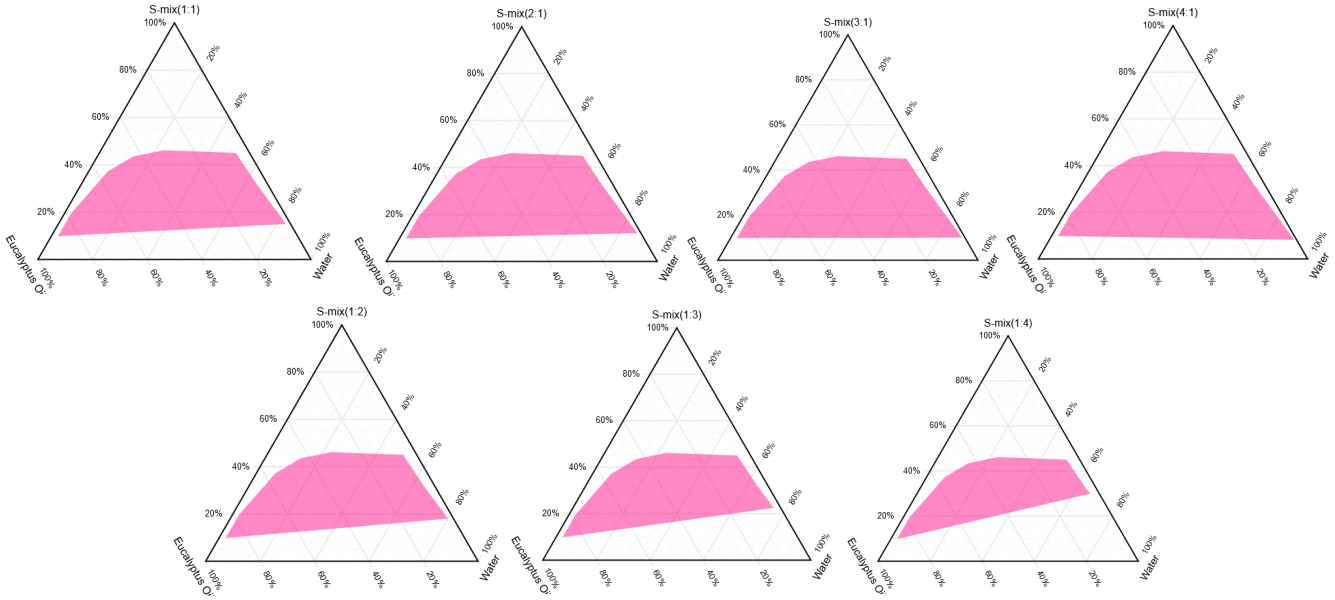

Figure 1: Pseudo ternary phase diagram of Eucalyptus oil, Tween 20, DMSO, and deionized water for different S-mix ratios.

Table 2: Solubility study of MTX

Oil	*Solubility (µg/mL)*	*Surfactant*	*Solubility (µg/mL)*	*Co-surfactant*	*Solubility (µg/mL)*
Eucalyptus	805.7	Tween 20	2833.8	DMSO	15000
Peppermint	787.9	Cremophor	2457.7	Transcutol P	1300
Cumin	708.5	Span 80	1187.3	Carbitol	1105
Anise	585.9	Tween 80	1070.2	Labrasol	1095
Argan	580.5	Triton x-114	74.1	PEG 200	1086
Cod Liver	580.1	Triton x-100	35.8	Propylene glycol	884.4
Olive	348.7			PEG400	580.1
Castor	246.5			Ethanol	56.7
Almond	240.3			Capryol	44.5
Triacetin	47.8			Iso amyl alcohol	27.1

Formulation of MTX-loaded Nanoemulsion

Fourteen nanoemulsion formulations loaded with MTX had a dose of 0.6 mg of MTX/1 g 0f nanoemulsion prepared by an aqueous titration method.

Thermodynamic Stability Studies of MTX Nanoemulsion Formulation

Thermodynamic stability tests were applied to all 14 formulations. The results showed all formulations were stable since no aggregation, flocculation, phase separation, or creaming occurred during all tests.

The great stability of prepared nanoemulsions could be attributed to the stabilization effect of surfactant tween 20 and appropriately chosen ratios of oil, surfactant, co-surfactant, and deionized water.[26]

Characterization of the Prepared MTX Nanoemulsion

Globule Size Measurement

The results of globule size were range from 57 nm for F 25 to 351 nm for F 18, as shown in Table 3. All prepared formulations had droplets size on a nanoscale.

Polydispersity Index (PDI) Study

PDI results were from (0.21 to 0.39) as shown in Table 3, which indicates that MTX nanoemulsion formulations had a great homogeneous and narrow size distribution.

Zeta Potential Study

Results of zeta potential measurement were in the range (+10.42 to +23.67 mV) as shown in Table 3, indicating respectable stability of MTX nanoemulsion formulations and excluding aggregation. Furthermore, tween 20 is a nonionic surfactant that has steric stability for protecting the prepared MTX nanoemulsions.

Determination of pH

The measured pH values of MTX nanoemulsion formulations ranged from 4.1 to 4.7, as shown in Table 3, which consider an accepted range.

Dilution Test Study

Clear homogenous solutions were achieved by diluting MTX nanoemulsion formulations with distilled water and phosphate buffer. No visual signs of turbidity, cracking, or phase separation were observed.

Electrical Conductivity Study

The measured conductivity values of MTX nanoemulsion formulations were in the range of 131 to 197 µs/cm (Table 3),

Table 3: results of the globule size distribution (GSD), polydispersity index (PDI), zeta potential, pH, electrical conductivity, percent of light transmittance (T%), and % of MTX content in the all nanoemulsion formulations (mean ± SD, n = 3).

F name	*GSD nm*	*PDI*	*Zeta p.*	*pH*	*E. C. (µs/cm)*	*T %*	*% MTX*
F 1	88	0.33	17.83	4.2	± 0.4 136 ± 0.8	97.25 ± 0.2	99.0 ± 0.3
F 2	74	0.29	11.45	4.3	± 0.2 143 ± 0.4	98.3 ± 0.3	97.7 ± 0.9
F 3	65	0.38	10.42	4.3	± 0.8 139 ± 0.8	99.3 ± 0.1	96.2 ± 0.5
F 4	61	0.22	10.65	4.1	± 0.3 145 ± 0.6	99.1 ± 0.2	95.7 ± 0.4
F 5	124	0.34	11.27	4.4	± 0.1 138 ± 0.8	97.5 ± 0.3	95.9 ± 0.8
F 6	158	0.31	16.75	4.3	± 0.2 151 ± 0.5	97.7 ± 0.2	96.8 ± 0.5
F 7	293	0.38	20.66	4.15	± 0.4 155 ± 0.4	98.4 ± 0.1	99.5 ± 0.2
F 8	96	0.25	14.2	4.5 ± 0.2	197 ± 0.4	99.4 ± 0.2	99.2 ± 0.3
F 9	77	0.29	19.5	4.6	± 0.4 188 ± 0.9	99.2 ± 0.1	96.1 ± 0.9
F 10	63	0.24	23.01	4.7	± 0.3 191 ± 0.2	97.3 ± 0.2	95.9 ± 0.3
F 11	57	0.21	23.67	4.15 ± 0.2	189 ± 0.9	99.57 ± 0.1	99.3 ± 0.3
F 12	135	0.27	12.87	4.5	± 0.3 190 ± 0.8	97.9 ± 0.2	96.5 ± 0.6
F 13	187	0.39	10.38	4.6	± 0.4 197 ± 0.6	99.3 ± 0.1	99.1 ± 0.2
F 14	351	0.35	10.86	4.7	± 0.2 191 ± 0.7	97.4 ± 0.2	95.5 ± 0.3

which indicates the o/w nature of the prepared MTX nanoemulsions since the external phase was water and it has high electrical conductivity.[27]

Transmittance Percent (T %) Measurement

The percent of light transmittance of all the prepared MTX nanoemulsions was found in the range (97.25-99.57%) as shown in Table 3, which means all formulations were transparent.

Dye Solubilization Study

Methyl orange (water-soluble dye) was spread homogenously with all MTX nanoemulsion formulations, confirming the construction of o/w nanoemulsion with aggregation.

Drug Content Estimation

The outcome results of drug content were 95.5–99.3%, as shown in Table 3. Hence it is set within USP officially accepted range.[28]

In-vitro Drug Release Study

Twelve MTX nanoemulsion formulations had globule sizes less than 200 nm chosen for the *in-vitro* release study, as shown in Figures 2 and 3.

The results reveal a flexible duration time for complete MTX released from each formula, in which F11 and F4 formulations completely released (100%) MTX after 30 minutes, while F 3, F 5, F 10, and F 12 needed 35 minutes for complete MTX release, other formulations (F 2, F 6, F 9, F13) and (F 1, F 8) showed nearly 100% MTX release after 40 and 45 minutes, respectively. Pure MTX dispersed in phosphate buffer was involved for the release study for comparison with nanoemulsion and exhibited a significantly ($p \leq 0.05$) slower release profile than all tested MTX nanoemulsion formulations, in which only 25.7% released after 50 minutes.

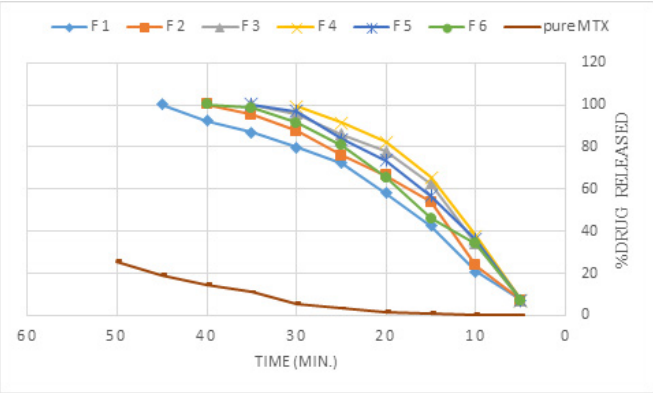

Figure 2: *In-vitro* release of MTX nanoemulsion formulations F 1, F 2, F 3, F 4, F 5, F 6 and pure MTX

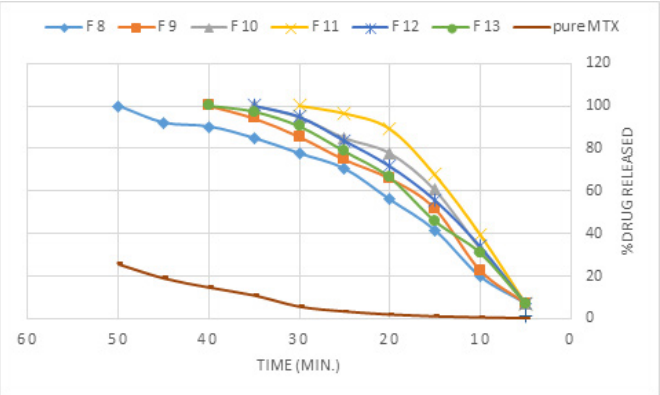

Figure 3: *In-vitro* release of MTX nanoemulsion formulations F 8, F 9, F 10, F 11, F 12, F 13, and pure MTX.

The release profile was greatly dependent on globule size, as a faster MTX release of 30 minutes was stated in F 11 and F 4 formulation with globule size less than 62 nm, while slower release profile of 45 minutes was exhibited by F 1 and F 8_with globule size near 100 nm. The effect of globule size on MTX release profile could be attributed to the obvious increase in effective interfacial area of MTX particles exposed to dissolution media (phosphate buffer), hence a higher rate of dissolution and rapid MTX release.[28,29] Concerning the influence of S-mix ratio, it was noticed that formulation contain higher S-mix ratios give faster MTX release profile due to solubilizing effect as well as hydrophilicity boost of MTX made by using tween 20 surfactant in high concentration.[30,31]

Selection of the Optimum Formula

Based on previous results, F 11_was chosen as the best MTX nanoemulsion formula since it had a globule size range of 57 nm, low PDI (0.21), high zeta potential (+23.67), respectable pH (4.15), good electrical conductivity (189 µs/cm), elegant T% (99.57), greater % drug content (99.3) and higher dissolution rate. The selected formula was further subjected to drug excipient compatibility study by FTIR spectroscopy.

Drug – Excipients Compatibility Study

The result of FTIR of pure MTX powder showed characteristic absorptions broadband signal at 3450 cm $^{-1}$ (o-H) stretching of carboxyl groups, at 3080 cm $^{-1}$ (N-H) stretching of primary amine, at 1670-1600 cm $^{-1}$ given by (C=O) stretching from carboxyl group and (C=O) stretching from amide group, so the band split into a doublet. The bands are equivalent to (N-H) bending of the amide group seen in the 1550–1500 cm^{-1} range. Other noticeable bands like 1400-1200 cm^{-1} represent (-C-O) stretching of carboxyl group, 930 cm^{-1} for (O-H) bending out of plane and 820 cm^{-1} for (C-H) adjacent hydrogens on an aromatic ring (para substituted) as shown in Figure 4.

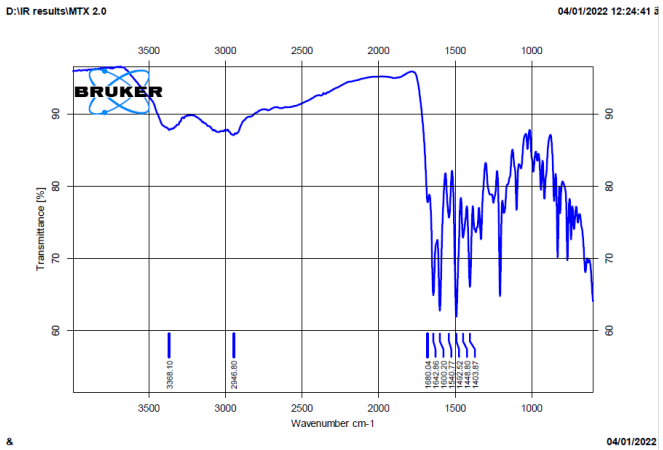

Figure 4: FTIR spectrum of pure MTX.

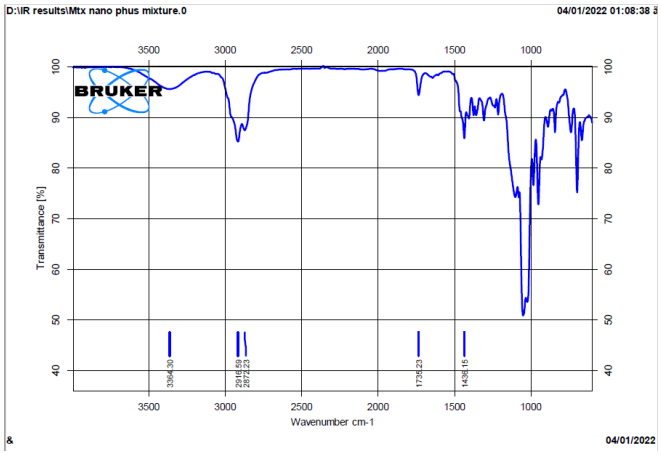

Figure 5: FTIR spectrum of (1:1) physical mixture.

These identified bands in the FTIR spectrum are in good agreement with the molecular chemical structure of MTX and approve its purity.[32]

The FTIR spectrum of physical mixture (1:1) of MTX with all ingredients of the selected formula (Eucalyptus oil, tween 20, and DMSO) demonstrated that the characteristic peaks of MTX are not affected and observed in the spectrum, which indicate no incompatibility presented between MTX and other excipients also no interaction occurred between MTX and any of component of nanoemulsion, as shown in Figure 5.

CONCLUSION

MTX was fruitfully formulated as o/w nanoemulsion with a globule size range less than 200 nm by carefully selected ratios of Eucalyptus oil, tween 20, DMSO, and distilled water. The prepared MTX nanoemulsion formulations exhibit significantly improved solubility, permeability, and bioavailability.

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