Formulation and Evaluation of O/W Nanoemulsions of Caffeic Acid

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Abstract

Objective: To Formulate and Evaluate Nanoemulsions of Caffeic acid to overcome its poor aqueous solubility and bioavailability.

Methods: The caffeic acid nanoemulsions were prepared by spontaneous emulsification method through selection of oil phase based on solubility studies and surfactant and co-surfactant screening on the basis of their emulsification ability. The components were selected on the basis of maximum solubility of caffeic acid in various media such as isopropyl myristate as oil phase, tween 80 as surfactant and PEG 600 as co-surfactant. Pseudo-ternary phase diagrams were constructed to optimize the percentage of oil, surfactant and co-surfactant. The prepared nanoemulsions were then evaluated for its surface morphology, zeta potential, droplet size, pH, viscosity and drug content.

Result: The formulated nanoemulsion showed -0.0468 mV zeta potential with droplet size below 100 nm, pH 5.9 and viscosity 9.3 cps. The *in-vitro* drug release studies showed 98.76% drug release in 0.1N HCl while 98.57% in phosphate buffer (pH 6.8) that was found to be significantly higher than that of drug.

Conclusion: The formulated nanoemulsions of caffeic acid showed improvement in solubility leading to enhanced oral bioavailability.

Keywords: Caffeic Acid, Nanoemulsions, Antioxidant Potential, Pseudo Ternary Phase

Introduction

Caffeic acid is a natural phytochemical isolated from various plants such as *Ilex paraguariensis*, *Melissa officinalis* and *Baccharis genistelloides* and bark of *Eucalyptus globulus*. It is a secondary metabolite obtained from vegetables (potato, carrot, cabbage, cauliflower and radish), fruits (strawberry, pear and apple), coffee beans and medicine named as Propolis [1]. It is a derivative of hydroxycinnamic acid that

has pharmacological activities like hepatoprotective, anti-proliferative [1], anticarcinogenic [4], antiviral, antidiabetic and anti-atherosclerotic [1] etc.

Owing to its antioxidant potential, it can be used as a photoprotective agent in dermal care products. It prevents formation of reactive oxygen species (ROS) thereby, reducing the oxidative stress. Caffeic acid has low aqueous solublility, low bioavailability and bitter taste that restricts its oral use [3]. So, to improve its solubility, bioavailability and specificity, it was formulated as nanoemulsions as it is a good approach to improve the solubility alongwith bioavailability.

Nanoemulsions (NEs) are the thermodynamically stable isotropic dispersions of oil, aqueous phase, surfactant and co-surfactant in suitable ratios. They can also be recognised as mini-emulsions, ultrafine emulsions or submicron emulsions. They have low interfacial tension, accomplished by addition of a co-surfactant [5]. Blend of surfactant and co-surfactant forms an interfacial film that stabilize molecules less than 100 nm. The dispersed phase comprises of small droplets having a size range 5 nm to 200 nm [6]. The small droplet size not only suppresses droplets coagulation but also deliver drug and avoids precipitation of nanoemulsions. They increase the drug solubility through core entrapment in nanoemulsion droplets and have potential for targeting tumour cells [7].

NEs enhance pharmacological and therapeutic potential of drug and thereby, reduce adverse reactions or toxic effects of drugs [8]. Owing to their small droplet size, they easily penetrate through the skin surface [9].

On the basis of composition, NEs are classified as [6]:
- Oil in water (o/w) nanoemulsions
- Water in oil (w/o) nanoemulsions
- Bicontinuous nanoemulsions (o/w/o) and (w/o/w)

Materials and Methods

Materials

Caffeic acid was purchased from Central Drug House (Delhi), Isopropyl myristate from Loba Chemie Pvt. Ltd., Tween 80 from Nice Chemicals, Polyethylene glycol from Nice Chemicals and distilled water from Organo Biotech. All other chemicals used were of analytical grade.

Methods

Determination of λ_{max} of Drug (in Methanol, 0.1N HCl, Phosphate Buffer pH 6.8)

To measure the absorption maxima of drug, a stock solution was prepared by dissolving 10 mg of drug in 10 ml of methanol, 0.1N HCl and 6.8 pH phosphate buffer separately to get 1000 µg/ml for each. 1.0 ml of this stock solution was diluted up to 10 ml with methanol, 0.1N HCl and 6.8 pH phosphate buffer respectively in order to obtain a concentration of 100 µg/ml. The absorption maxima at 200-400 nm were recorded with the help of UV spectrophotometer by using methanol, 0.1N HCl and 6.8 pH phosphate buffer as a reference solution.

Preparation of Calibration Curve

- **In Methanol:** Dilutions were made from stock solution in a volumetric flask. The absorbance of these dilutions was measured at 217 nm with UV spectrophotometer using methanol as reference.

- **In 0.1N HCl:** The absorbance of different dilutions was measured at 322 nm with the help of UV spectrophotometer using 0.1N HCl as reference.
- **In Phosphate Buffer pH 6.8:** The absorbance of these different dilutions was measured at 216 nm with the help of UV spectrophotometer using phosphate buffer as reference.

The calibration curve was then plotted.

Solubility Studies

Dissolve excess of caffeic acid (100 mg) in 5 ml of each of the selected vehicle, i.e. oil, co-surfactant and surfactant in stoppered glass vials separately. Then, the mixture was vortexed for 10 minutes and sonicated using probe sonicator for 8 minutes in order to facilitate proper mixing and reduce the particle size. The mixtures were then kept at 37 ± 1.0 °C in a shaker bath for 72 hours to get equilibrium.

The equilibrated samples were removed from shaker and were centrifuged at 3000 rpm for 15 minutes. The supernatant was taken and filtered through 0.45 µm membrane filter to remove the remaining caffeic acid. Further, 1 ml of this filtrate was diluted up to 10 ml with methanol and caffeic acid concentration in the filtrate was determined at 217 nm using UV spectrophotometer. The solubility of caffeic acid in different oils, surfactants and co-surfactants was determined with the help of standard calibration curve of drug in methanol [10] [11].

Screening of Optimized Ratio of Oil, Surfactant and Co-Surfactant

On the basis of solubility studies oil, surfactant and co-surfactant were selected for formulation. The selected components were optimized for their emulsifying ability to form nanoemulsion by carrying out the screening procedure (% transparency).

In a glass vial, the oil was mixed with surfactant and co-surfactant in a fixed ratio and mixture was heated at 40 ºC for 30 seconds. Then mixture was vortexed for 3 minutes and added drop by drop in 200 ml of distilled water and kept for 2 hours. The % transparency was measured at 217 nm with the help of UV spectrophotometer. The combination which showed good % transmittance was further optimized by constructing pseudo ternary phase diagrams [12].

Optimization of Aqueous Phase Concentration for Nanoemulsions using Pseudo Ternary Phase Diagram

The pseudo-ternary phase diagrams were constructed to determine the region of nano-emulsification, that had the highest probability of forming a transparent nanoemulsion and to optimize the percentage of oil, surfactant and co-surfactant for the formulation.

Pseudo ternary phase diagrams were constructed by using titration method to obtain the o/w nanoemulsion region and the concentration of the components (oil, surfactant and co-surfactant) was identified. The ratio of weight of surfactant to cosurfactant (K_m) was varied as 1:1, and 2:1 and the ratio of oil: surfactant/ co-surfactant was varied as 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1 respectively. The required amount of the three components (oil, surfactant and co-surfactant) were weighed accurately and vortexed for 3 minutes. Then, water was added dropwise to each oily mixture under proper magnetic stirring at 37 ºC so that the mixture became clear and transparent at a certain point. Then the concentration of the components was used to plot the pseudo-ternary phase diagram [12] [13].

Structural Compatibility Studies between Drug and Excipients

FT-IR analysis was carried out to assess the drug and excipient interactions. FT-IR of pure drug and a mixture of drug with excipients (blend of surfactant, cosurfactant and oil) was carried out for qualitative identification of compound. Caffeic acid and its mixtures were analyzed over the range 4000-400 cm^{-1}.

Preparation of Nanoemulsion

Nanoemulsions were prepared by using isopropyl myristate as oil, tween 80 as surfactant, PEG 600 as co-surfactant along with aqueous phase. With the help of pseudo-ternary phase diagram existing NE region was found and concentration of oil, surfactant and co-surfactant at appropriate weight ratios were selected for formulation.

Accurately weighed caffeic acid was added to adequate amount of oil in a cleaned and dry vial and was vortexed. Then to the mixture, surfactant and co-surfactant at definite ratio (S_{mix}) were added and again the mixture was vortexed. The above mixture was finally allowed to titrate by distilled water under vortex mixer. Then, the mixture was sonicated for 3 minutes using probe sonicator. The mixture was stored at room temperature for further use [14].

Evaluation of Nanoemulsion

1. **Morphological Evaluation**: Few drops of nanoemulsion prepared in double distilled water were placed onto holey film grid and immobilized. The excess of solution was wicked off from the grid followed by immobilization and staining. The stained nanoemulsions were then examined for its surface morphology and structure [15].
2. **Droplet Size Analysis**: Droplet size of NEs was determined by photon correlation spectroscopy using Malvern zetasizer ver. 7.12, that analyzed the fluctuation in scattering of light due to brownian motion. The sample was sonicated prior to droplet size determination [8].
   The formulation was dispersed in double distilled water to obtain homogeneous dispersion and used instantly for measuring the droplet size. The droplets showed random movement in a liquid and the speed at which they move was utilized to measure droplet size [16].
3. **Polydispersity Index (PI)**: Polydispersity of NEs were analyzed by employing photon correlation spectroscopy using malvern zetasizer ver.7.12. It indicated the uniformity of droplet size in formulation and it varies from 0.0 to 1.0. The higher value of polydispersity indicated the lower uniformity of droplet size in formulation [17].
4. **Zeta Potential**: Zeta potential determined the physical stability of the nanoemulsion. It was quantified as particle charge which was measured by electrophoretic mobility of particles in an electrical field. Zeta potential of NEs was measured by Malvern zetasizer ver. 7.12. For measuring zeta potential, NE was diluted and its value was estimated from the electrophoretic mobility of oil droplets. Zeta potential of ± 30 mV was sufficient for ensuring physical stability of nanoemulsion [15].
5. **pH:** The digital pH meter was used for measuring the pH of the nanoemulsion [15].
6. **Refractive Index:** Refractive index was determined by Abbes refractometer at 25 ± 0.5 °C, by placing a drop of nanoemulsion on slide and compared with refractive index of water (1.333). If refractive index of nanoemulsion was found to be equal as that of water, then it was considered of transparent nature [15].
7. **Determination of Viscosity:** The viscosity of the formulation was determined as such without dilution using brookfield viscometer at 25 ± 0.5 ºC. The speed of the spindle, L3 was adjusted at 200 rpm. The viscosity confirmed the system whether it was o/w or w/o emulsion. The formulation having

low viscosity showed o/w type emulsion whereas, high viscosity showed w/o type. Results were taken in triplicate and the average was considered [15].

8. **Determination of % Drug Entrapment:** The glass vial containing NE was sonicated for 3 minutes and then mixture was shaken for 72 hours at 37 ºC using flask shaker. The mixture was centrifuged at 12000 rpm for 10 minutes and 1ml of supernatant was taken and diluted with methanol and absorbance was measured at 217 nm by UV spectrophotometer. The concentration of caffeic acid was determined using standard curve equation and % drug entrapment was calculated using formula [15].

$$\% \text{ Drug Entrapment} = \text{Practical value} \div \text{Theoretical value} \times 100$$

9. **Physical Evaluation of Nanoemulsions:** The formulation was evaluated for determination of type of nanoemulsion i.e. o/w type by using parameters such as dilution with water, dye solubilization and its spreadability on filter paper.
   - **Dye Solubility Test:** Dye test was used to measure the colour uniformity of nanoemulsion. In this, the water-soluble dye, eosin yellow was added to 1 ml of nanoemulsion in an eppendrof tube and mixed properly. This was visualized with microscopic examination of nanoemulsion [16].
   - **Dilution Test:** Dilution test was performed in order to observe the phase inversion of the nanoemulsion. For this, 1 ml of nanoemulsion was diluted with 10 ml of water in a test tube and observed for phase inversion [16].
   - **Filter Paper Test:** This test was performed to determine the type of nanoemulsion. A drop of nanoemulsion was poured over the filter paper. If it was an o/w nanoemulsion, it will spread out rapidly when dropped onto filter paper whereas, if it was a w/o nanoemulsion it migrated slowly. This method was not suitable for highly viscous creams [8].
10. ***In-vitro* Drug Release Studies:** The *in-vitro* drug release was performed using USP dissolution apparatus II paddle assembly at 100 rpm at 37 ± 0.5 ºC. The formulation was tested individually in 0.1N HCl (pH 1.2) and in phosphate buffer (pH 6.8). These media were selected to mimic the physiological conditions in stomach and small intestine respectively. Aliquot samples were withdrawn at specified time intervals and each time replaced by the same volume of fresh dissolution medium. Samples were then diluted and analyzed spectrophotometrically at 322 and 216 nm respectively. The absorbance of the collected sample was used for determination of % drug release at different time intervals using calibration curve [15].
11. **Accelerated Stability Studies:** To assess the formulation stability, studies were performed as per ICH and WHO guidelines. The optimized formulation was stored at accelerated conditions of 40º ± 2 ºC and 75 ± 5% relative humidity (RH) in closed glass vials for 6 weeks. Samples were withdrawn and analyzed at specified time intervals 0, 2, 4, 6 weeks for any change in transparency, % drug content and *in-vitro* drug release.

Results and Discussion

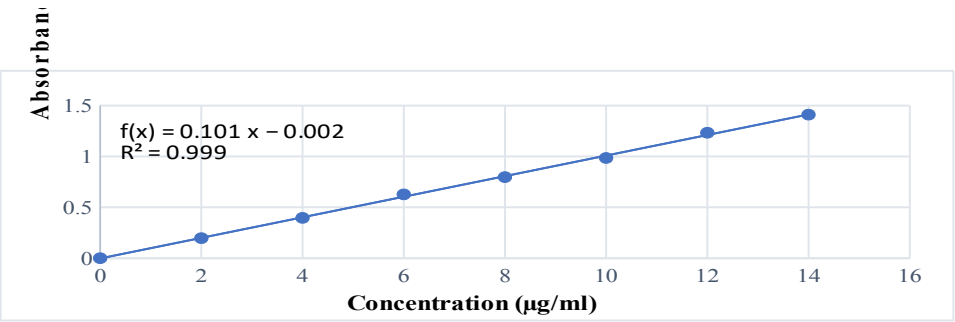
Preparation of Standard Calibration Curve In Methanol

Table 1: Absorbance in Methanol

Sr. No.	Concentration (µg/ml)	Absorbance (Mean ± SD)
1	0	0.000 ± 0.00
2	2	0.197 ± 0.02

3	4	0.396 ± 0.0.2
4	6	0.627 ± 0.03
5	8	0.7967 ± 0.03
6	10	0.985 ± 0.02
7	12	1.233 ± 0.02
8	14	1.411 ± 0.01

Figure 1: Standard Calibration Curve in Methanol



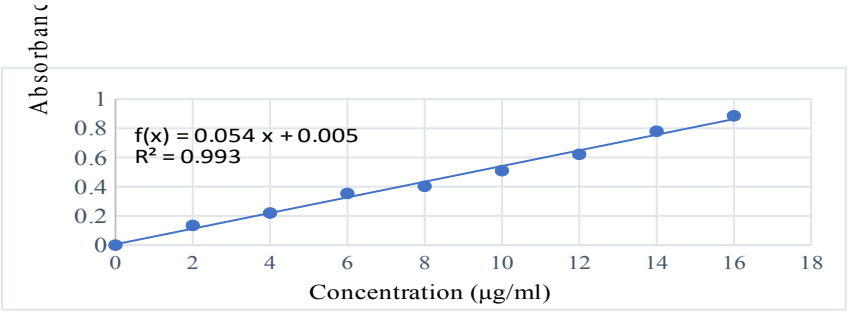
The standard curve shown in Figure 1 indicates good linearity.

In 0.1N HCl

Table 2: Absorbance at Different Concentration in 0.1N Hcl (n = 3)

Sr. No.	Concentration (µg/ml)	Absorbance (Mean ± SD)
1	0	0.000 ± 0.00
2	2	0.135 ± 0.03
3	4	0.22 ± 0.07
4	6	0.354 ± 0.03
5	8	0.403 ± 0.07
6	10	0.51 ± 0.05
7	12	0.622 ± 0.07
8	14	0.78 ± 0.07
9	16	0.886 ± 0.04

Figure 2: Standard Calibration Curve in 0.1N HCl



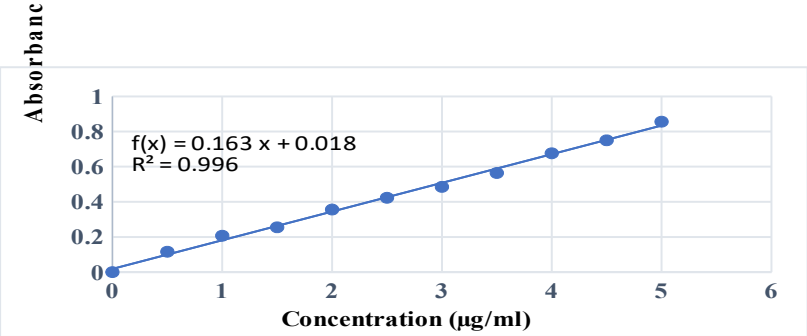
The standard curve shown in figure 2 indicates good linearity.

In Phosphate Buffer (pH 6.8)

Table 3: Absorbance at Different Concentration in Phosphate Buffer (pH 6.8) (n = 3)

Sr. No.	Concentration (µg/ml)	Absorbance (Mean ± SD)
1	0	0.000 ± 0.00
2	0.5	0.116 ± 0.02
3	1	0.207 ± 0.02
4	1.5	0.255 ± 0.02
5	2	0.356 ± 0.03
6	2.5	0.423 ± 0.02
7	3	0.485 ± 0.02
8	3.5	0.564 ± 0.01
9	4	0.676 ± 0.03
10	4.5	0.750 ± 0.07
11	5	0.856 ± 0.02

Figure 3: Standard Calibration Curve in Phosphate Buffer (pH 6.8)



The standard curve shown in Figure 3, indicated good linearity.

Solubility Studies

Solubility of caffeic acid in different oils, surfactants and co-surfactants was determined and results were shown in Table 4.

Table 4: Solubility of Caffeic Acid in Oil, Surfactant and Co-surfactant (n = 3)

Excipients	Solubility (mg/ml)
Oil	
Isopropyl Myristate	10.65 ± 0.001
Oleic Acid	5.41 ± 0.01
Castor Oil	2.55 ± 0.03

Surfactant	
Tween 80	11.45 ± 0.003
Span 20	4.38 ± 0.02
Span 80	1.33 ± 0.005
Co-surfactant	
Propylene Glycol	3.05 ± 0.006
PEG 600	9.41 ± 0.002
Glycerine	1.12 ± 0.005

On the basis of solubility, oil (Isopropyl myristate, oleic acid), surfactant (tween 80, span 20) and co-surfactant (PEG 600 and propylene glycol) were selected and a transparency study was done to determine compatibility between oil, surfactant and co-surfactant.

Screening of the Optimized Ratio of Oil, Surfactant and Co-surfactant

Determination of Transparency between Oil, Surfactant and Co-surfactant

The selected oils, surfactants and co-surfactants were screened to find the best combination. During screening, oil and surfactant/ co-surfactant mixture (S_{mix}) were taken in ratio 1:1 to determine the transparency.

Table 5: Transparency between Isopropyl Myristate, Tween 80 and Propylene Glycol (n = 3)

Sr. No.	Component	Oil: S_{mix}	% Transparency (Mean ± SD)
1	Isopropyl Myristate	1:1	65.66 ± 0.07
2	Tween 80		
3	Propylene Glycol		

Table 6: Transparency between Oleic Acid, Tween 80 and Propylene Glycol (n = 3)

Sr. No.	Component	Oil: S_{mix}	% Transparency (Mean ± SD)
1	Oleic Acid	1:1	44.37 ± 0.25
2	Tween 80		
3	Propylene Glycol		

Table 7: Transparency between Oleic Acid, Tween 80 and PEG 600 (n = 3)

Sr. No.	Component	Oil: S_{mix}	% Transparency (Mean ± SD)
1	Oleic Acid	1:1	34.68 ± 0.15
2	Tween 80		
3	PEG 600		

Table 8: Transparency between Isopropyl Myristate, Tween 80 and PEG 600 (n = 3)

Sr. No.	Component	Oil: S_{mix}	% Transparency (Mean ± SD)
1	Isopropyl Myristate	1:1	87.39 ± 0.04
2	Tween 80		
3	PEG 600		

Table 9: Transparency between Isopropyl Myristate, Span 20 and PEG 600 (n = 3)

Sr. No.	Component	Oil: S_{mix}	% Transparency (Mean ± SD)
1	Isopropyl Myristate	1:1	39.45 ± 0.07
2	Span 20		
3	PEG 600		

Table 10: Transparency between Oleic Acid, Span 20 and Propylene Glycol (n = 3)

Sr. No.	Component	Oil: S_{mix}	% Transparency (Mean ± SD)
1	Oleic Acid	1:1	22.51 ± 0.09
2	Span 20		
3	Propylene Glycol		

On the basis of transparency, components of nanoemulsion (isopropyl myristate as oil, tween 80 as surfactant and PEG 600 as a co-surfactant) were selected because of maximum transparency (87.39%) between them.

A value of percentage transmittance closer to 100% indicated that the optimized formulation was clear and transparent.

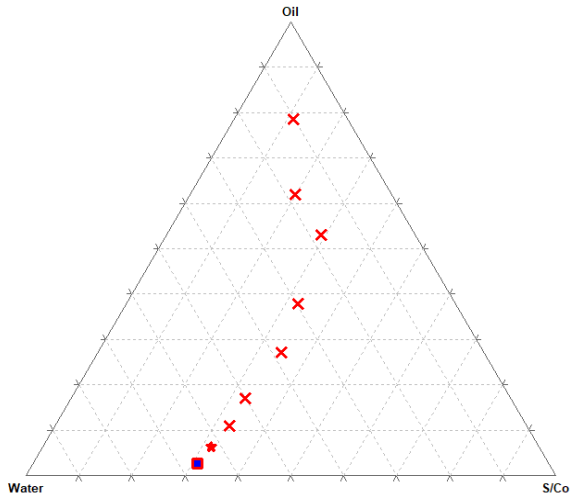
Optimization of Aqueous Phase Concentration for NEs using Pseudo Ternary Phase Diagram

Pseudo-ternary phase diagram was used to obtain the o/w nanoemulsion region which involves stepwise addition of water to each weight ratio of oil and surfactant mixture, and then mixing the components with the help of vortex mixer. The ratio of surfactant (tween 80) to co-surfactant (PEG 600) was varied as 1:1 and 2:1 and the ratio of oil: surfactant/co-surfactant was varied as 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1. From the end point, compositions of the titrated samples, the mass percent compositions of isopropyl myristate, surfactant and co-surfactant mixture (S_{mix}) and water were calculated and plotted on triangular coordinates to construct the pseudo ternary phase diagrams using triplot software.

The nanoemulsion phase was identified as clear and transparent region in the phase diagram based on the visual observation where one axis of the pseudo-three component phase diagram represented the aqueous phase, the other represented the oil phase and the third represented a mixture of surfactant and co-surfactant at a fixed weight ratio (S_{mix}).

Tween 80: PEG 600:: 1:1

Figure 4: Ternary Phase Diagram (K_m = 1:1)



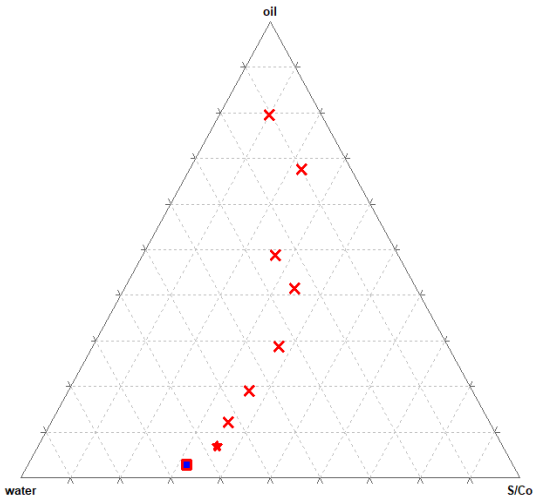
Ternary phase diagram plot shown in Figure 5.11, when surfactant: co-surfactant (K_m) is 1:1. It represents a three-component system {oil, water and K_m (surfactant + co-surfactant)}. The symbol (■) represented NE region (transparent and clear) and other symbols represented coarse emulsion (turbid). The NE region depends upon transparent nature after titration with water (0.05 ml water was added at a time).

Table 11: Degree of Transparency of Various Formulations (when K_m = 1:1)

Sr. No.	Oil:S/Cos	Formulation Code	Appearance	Observation
1	1:9	A1	Clear and Transparent	NE
2	2:8	A2	Turbid	Coarse Emulsion
3	3:7	A3	Turbid	Coarse Emulsion
4	4:6	A4	Turbid	Coarse Emulsion
5	5:5	A5	Turbid	Coarse Emulsion
6	6:4	A6	Turbid	Coarse Emulsion
7	7:3	A7	Turbid	Coarse Emulsion
8	8:2	A8	Turbid	Coarse Emulsion
9	9:1	A9	Turbid	Coarse Emulsion

Tween 80: PEG 600::2:1

Figure 5: Ternary Phase Diagram (K_m = 2:1)



Ternary phase diagram plot shown in Figure 5.12, when surfactant: co-surfactant (K_m) is 2:1. It represents a three-component system {oil, water and K_m (surfactant + co-surfactant)}. The symbol (■ and ★) represented NE region (transparent and clear) and other symbols represented coarse emulsion (turbid). The NE region depends upon transparent nature after titration with water (0.05 ml water was added at a time).

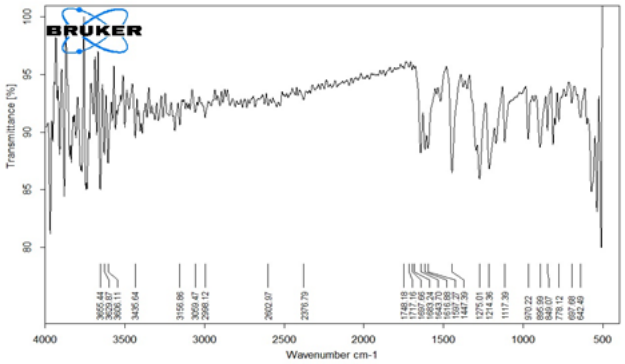
Table 12: Degree of Transparency of Various Formulations (when K_m = 2:1)

Sr. No.	Oil:S/Cos	Formulation Code	Appearance	Observation
1	1:9	B1	Clear And Transparent	NE
2	2:8	B2	Clear And Transparent	NE
3	3:7	B3	Turbid	Coarse Emulsion
4	4:6	B4	Turbid	Coarse Emulsion
5	5:5	B5	Turbid	Coarse Emulsion
6	6:4	B6	Turbid	Coarse Emulsion
7	7:3	B7	Turbid	Coarse Emulsion
8	8:2	B8	Turbid	Coarse Emulsion
9	9:1	B9	Turbid	Coarse Emulsion

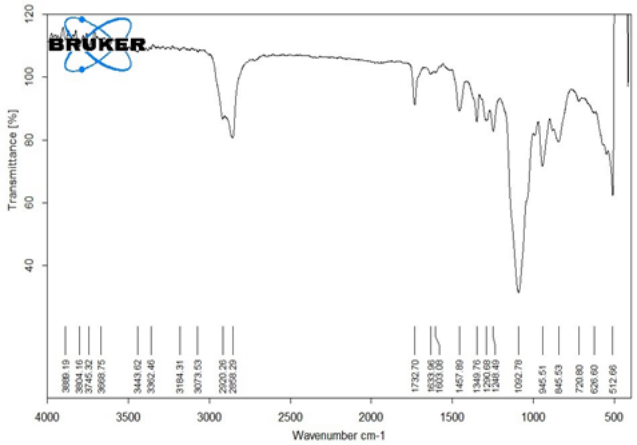
Drug Excipient Compatibility Study

Figure 6: FT-IR Spectra of (a) Caffeic Acid, and (b) Nanoemulsions of Caffeic Acid

a



b



The FT-IR spectra of caffeic acid and nanoemulsions of caffeic acid were shown in Figure 5. The spectrum of caffeic acid nanoemulsions showed characteristic peak at 1732 cm^{-1} (-C=O), 3443 cm^{-1} (OH-C=O), 3073 cm^{-1} (aromatic C-H stretching), 1457 cm^{-1} (aromatic C=C), 1633 cm^{-1} (C=C stretching), 1248 cm^{-1} (C-O stretching) and 845 cm^{-1}, 720 cm^{-1}, 626 cm^{-1}. It was concluded that there were no significant changes in the position of characteristic peaks of the drug when mixed with oil, surfactant and co-surfactant, which indicated compatibility of drug and the excipients.

Preparation of Nanoemulsion

Table 13: Composition of Nanoemulsion

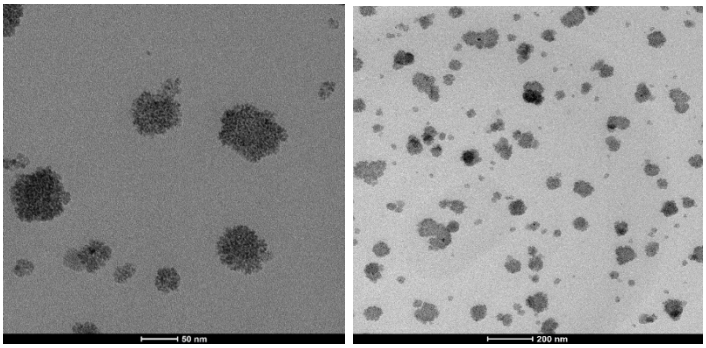
Sr. No.	Ingredient	%w/w
1	Isopropyl Myristate	2.63
2	S_{mix} (Tween 80: PEG 600)	65.12
3	Distilled Water	32.25

Evaluation of Nanoemulsions

1. Transmission Electron Microscopy

It was observed that the particles were uniformly distributed and were spherical in shape with size less than 100 nm. The results confirmed that the droplets were discrete and non-aggregated [16].

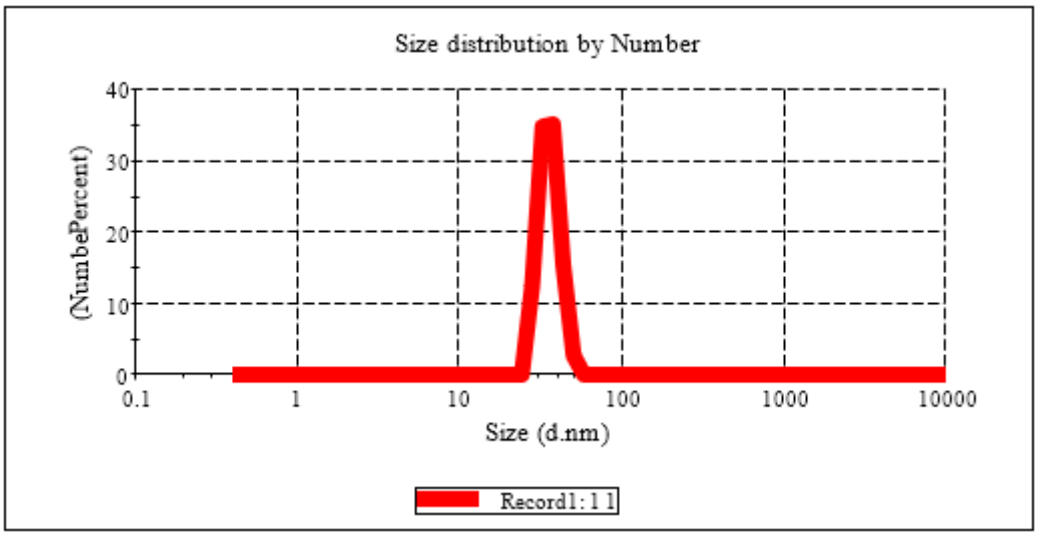
Figure 7: TEM Images of Nanoemulsion



2. Droplet Size Analysis

Small droplet size prevented the flocculation so that the droplets remain dispersed without separation [16].

Figure 8: Graphical Representation of Droplet Size Distribution



Droplet size of the caffeic acid nanoemulsion was found to be 36.12 d.nm. Thus, the results showed the droplet size in the desirable range i.e. less than 100 nm.

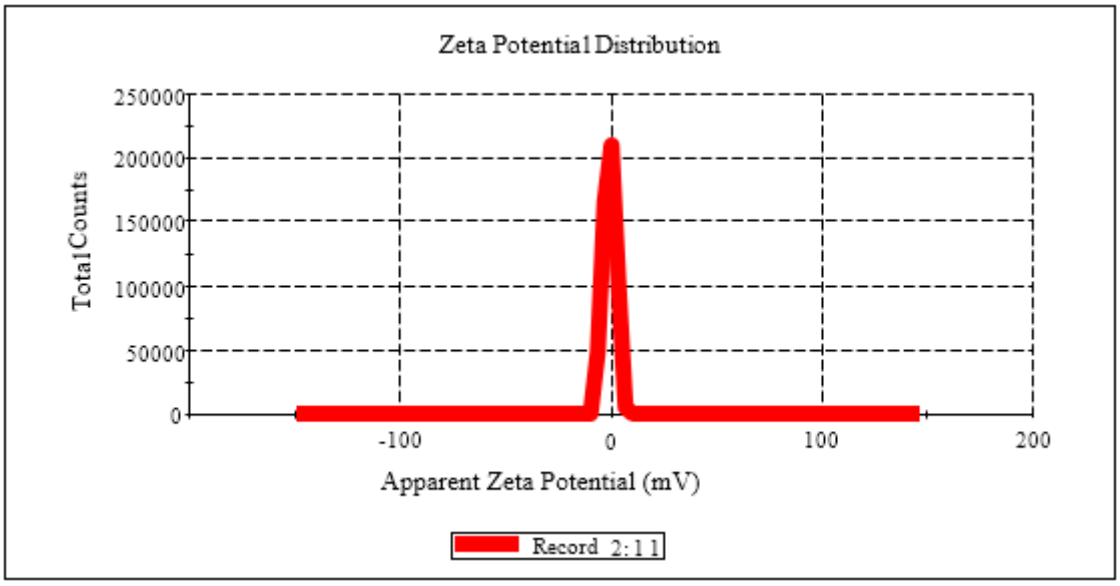
3. Polydispersity Index

The polydispersity value closer to zero showed that the particles were homogeneous. The PI value of the caffeic acid nanoemulsion was found to be PI 0.278 [16].

4. Zeta Potential

Zeta potential signified the charge on the droplets that indicated the degree of repulsion between the like charged particles. The more negative charge of zeta potential had greater net charge on droplets and thus, greater stability.

Figure 9: Graphical Representation of Zeta Potential



Zeta potential of the caffeic acid nanoemulsion was found to be -0.0468 mV which indicated the electrostatically stabilized nanoemulsion. The negative charge of the formulation was due to the anionic groups of the fatty acids and glycols present in the surfactant and co-surfactant. Thus, there are minimal chances of aggregation of nanoemulsion [16].

5. pH

The pH of the formulation was found to be 5.9 ± 0.17 by using digital pH meter at 25º ± 1 ºC.

6. Refractive Index

The refractive index of caffeic acid nanoemulsion was determined using an Abbes refractometer. If refractive index of nanoemulsion was found to be approximately equal as that of water, then it was considered to have transparent nature [18]. It was found to be 1.39 ± 0.08 which indicated the isotropic nature of the nanoemulsion.

7. Viscosity Determination

For determination of viscosity, spindle no. L3 was used and the viscosity was low and was found to be 9.3 cps at 27.6 ºC.

8. Determination of % Drug Entrapment

The % drug entrapment of the formulation was found to be 92.70 ± 0.07.

9. Physical Evaluation of Nanoemulsions

- **Dye Solubility Test**

Eosin yellow dye was added to nanoemulsions and observed under microscope. It was found that the continuous aqueous phase was evenly distributed with dye whereas, the dispersed oily phase remained undistributed. This confirmed that the nanoemulsion was o/w type [16].

- **Dilution Test**

Dilution test was performed and observed for phase inversion. The nanoemulsion was diluted with distilled water in the ratio 1:10, 1:50, 1:100. The nanoemulsion did not showed any sign of phase inversion. Thus, this test confirmed that the nanoemulsion was stable [16].

- **Filter Paper Test**

The nanoemulsion was dropped onto filter paper that indicate rapid spreadability over filter paper due to the aqueous nature of continuous phase. This confirmed the presence of o/w nanoemulsion [19].

10. *In-vitro* Drug Release Studies

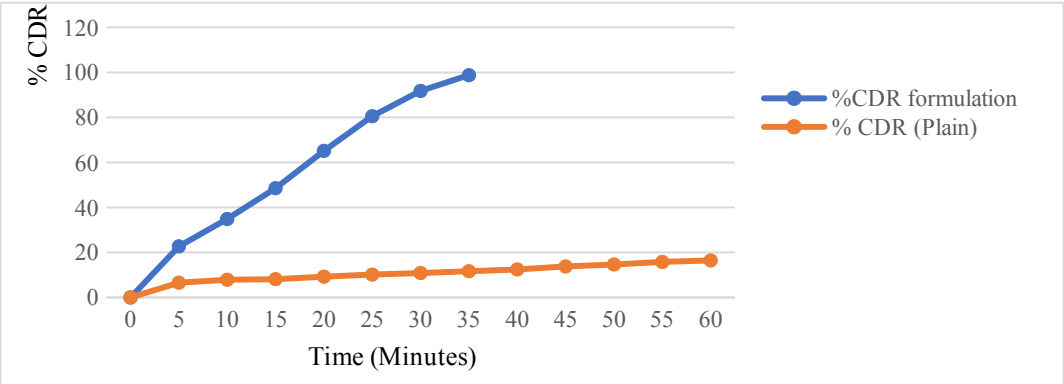
The dissolution study was performed using USP dissolution apparatus II paddle assembly in 900 ml of 0.1N HCl and phosphate buffer (pH 6.8) at 100 rpm at 37 ± 0.5 ºC [16].

a. Drug Release Study in 0.1N HCl

Table 14: % CDR of Formulation and Caffeic Acid in 0.1N HCl (n = 3)

Sr. No.	Time (Minutes)	% CDR (Formulation) (Mean ± SD)	% CDR (Caffeic Acid) (Mean ± SD)
1	0	0 ± 0.00	0 ± 0.00
2	5	22.70 ± 1.09	6.56 ± 0.08
3	10	34.86 ± 1.34	7.88 ± 0.98
4	15	48.54 ± 1.65	8.12 ± 0.67
5	20	65.14 ± 1.66	9.23 ± 0.56
6	25	80.54 ± 0.67	10.17 ± 0.57
7	30	91.78 ± 0.89	10.87 ± 0.78
8	35	98.76 ± 0.09	11.67 ± 0.77
9	40	-	12.45 ± 0.65
10	45	-	13.77 ± 1.06
11	50	-	14.65 ± 1.09
12	55	-	15.77 ± 1.12
13	60	-	16.45 ± 0.64

Figure 10: Comparative Results of % CDR from Formulation and Caffeic Acid in 0.1N HCl



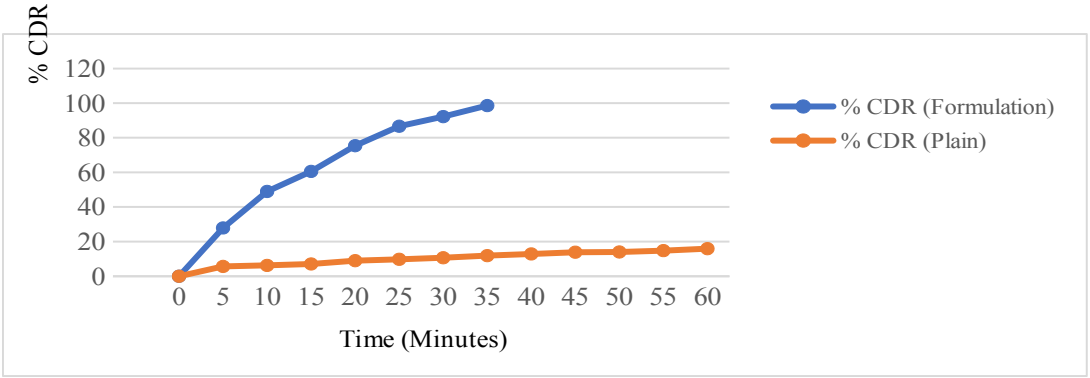
b. *In-vitro* dissolution in phosphate buffer (pH 6.8)

Table 15: % CDR of Formulation and Caffeic Acid in Phosphate Buffer (n = 3)

Sr. No.	Time (Minutes)	% CDR (Formulation) (Mean ± SD)	% CDR (Caffeic Acid) (Mean ± SD)
1	0	0 ± 0.00	0 ± 0.00
2	5	27.89 ± 0.87	5.67 ± 0.28
3	10	48.98 ± 0.56	6.26 ± 0.22
4	15	60.55 ± 0.57	7.09 ± 0.14
5	20	75.41 ± 0.78	8.99 ± 0.53

6	25	86.65 ± 0.88	9.78 ± 0.44
7	30	92.20 ± 0.99	10.67 ± 0.58
8	35	98.57 ± 0.15	11.90 ± 0.67
9	40	-	12.88 ± 0.32
10	45	-	13.89 ± 0.78
11	50	-	14.02 ± 0.88
12	55	-	14.79 ± 0.23
13	60	-	15.92 ± 0.70

Figure 11: Comparative Results of % CDR from Formulation and Caffeic Acid in Phosphate Buffer



It was found that drug release from the formulation was found to be higher as compared with that of caffeic acid as more than 90% drug released in both the dissolution medium i.e. 0.1N HCl and phosphate buffer (pH 6.8) in 35 minutes whereas only 16.45% drug released in 0.1N HCl and 15.92% drug released in phosphate buffer (pH 6.8) in 60 minutes. This was due to the small droplet size which provided large surface area for release of drug.

Thus, the faster rate of dissolution of caffeic acid nanoemulsion lead to higher absorption and higher oral bioavailability.

11. Accelerated Stability Studies

Accelerated stability studies were performed as per ICH and WHO guidelines. The formulation was stored at 40º ± 2 ºC and 75 ± 5% RH in closed glass vials for 6 weeks. The samples were withdrawn and analyzed at specified time intervals (0, 2, 4 and 6 weeks) for any change in the transparency, drug entrapment and the degree of dissolution [20] [21].

Table 16: Transparency and Drug Entrapment in Formulation at Different Time Interval (n = 3)

Sr. No.	Time (Weeks)	% Transparency	% Drug Entrapment
1	0	92.54	92.70
2	2	91.01	91.89
3	4	90.84	91.04

4	6	90.09	90.68

In-vitro Drug Release Studies

In-vitro drug release study was carried out for the formulation at time intervals (0, 2, 4 and 6 weeks) to determine the drug release from formulation at accelerated temperature conditions in 0.1N HCl and phosphate buffer separately [16].

Table 17: Drug Release Profile of Formulation in 0.1N HCl at Different Time Interval (n = 3)

Sr. No.	Time (Minutes)	% CDR			
		0	2	4	6
1	0	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
2	5	22.70 ± 1.09	22.56 ± 1.09	22.23 ± 0.08	20.89 ± 0.87
3	10	34.86 ± 1.34	33.90 ± 0.09	33.87 ± 0.76	32.09 ± 0.06
4	15	48.54 ± 1.65	48.12 ± 0.99	48.01 ± 0.99	46.99 ± 0.90
5	20	65.14 ± 1.66	64.76 ± 0.13	64.12 ± 0.06	63.01 ± 0.23
6	25	80.54 ± 0.67	80.12 ± 0.88	79.97 ± 0.32	78.23 ± 0.12
7	30	91.78 ± 0.89	91.08 ± 0.07	90.98 ± 0.76	89.87 ± 0.76
8	35	98.76 ± 0.09	98.06 ± 0.05	97.57 ± 0.43	96.44 ± 0.15

Figure 12: % CDR of Caffeic Acid from Formulation in 0.1N HCl

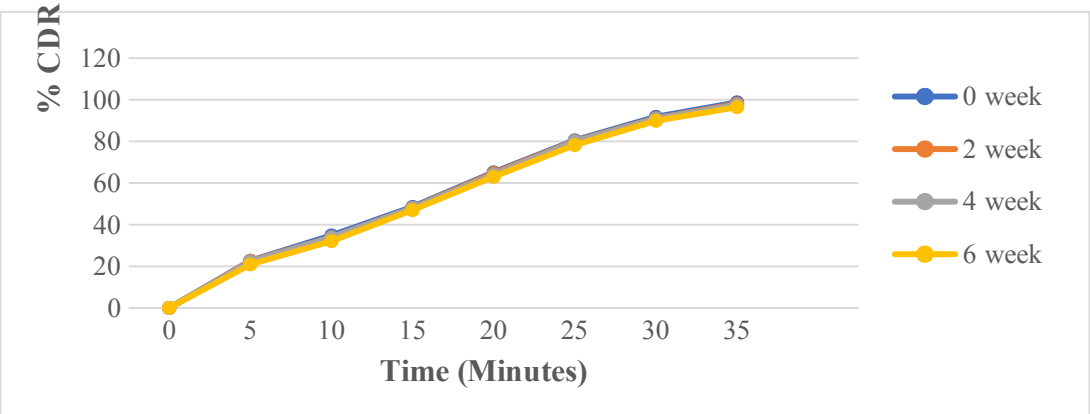
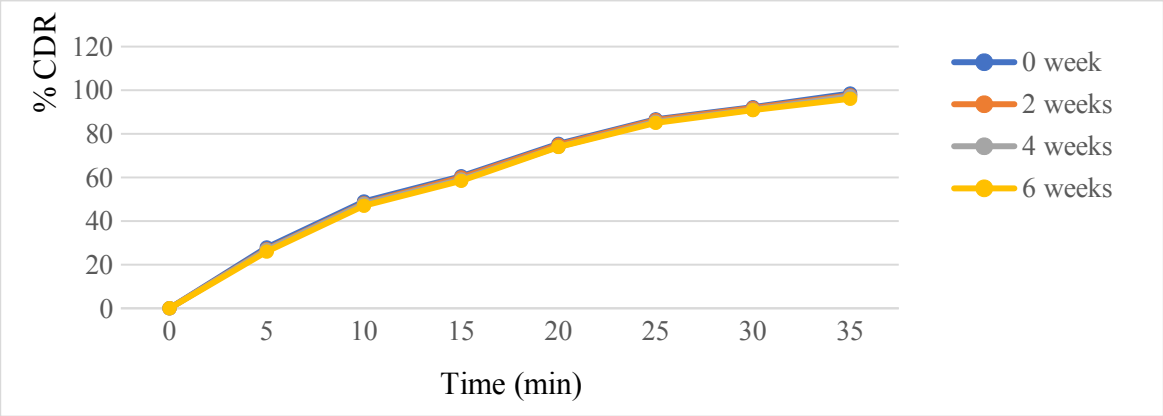


Table 18: Drug Release Profile of Formulation in Phosphate Buffer (n = 3)

Sr. No.	Time (Minutes)	% CDR			
		0 Week	2 Weeks	4 Weeks	6 Weeks
1	0	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
2	5	27.89 ± 0.87	27.02 ± 0.09	26.77 ± 0.67	25.99 ± 0.34
3	10	48.98 ± 0.56	48.12 ± 0.87	47.87 ± 0.54	47.01 ± 0.09
4	15	60.55 ± 0.57	59.99 ± 0.65	59.07 ± 0.76	58.44 ± 0.31
5	20	75.41 ± 0.78	74.89 ± 0.34	74.03 ± 0.05	73.98 ± 0.11

6	25	86.65 ± 0.88	86.31 ± 0.01	85.57 ± 0.64	85.05 ± 0.32
7	30	92.20 ± 0.99	91.87 ± 0.85	91.14 ± 0.34	90.86 ± 0.09
8	35	98.57 ± 0.15	97.67 ± 0.11	97.22 ± 0.35	96.09 ± 0.45

Figure 13: % CDR of Caffeic Acid from Formulation at Different Time Interval in Phosphate Buffer



The optimized formulation was analyzed for transparency, drug entrapment and degree of dissolution in 0.1N HCl and phosphate buffer (pH 6.8) at specific time interval (0, 2, 4, 6 weeks). No major change in transparency and drug entrapment was observed. In dissolution study, more than 90% drug released in 35 minutes in both of the dissolution medium at time interval (0, 2, 4 and 6 weeks) was observed. Hence, it was concluded that optimized formulation was stable at accelerated temperature condition.

Conclusion

Caffeic acid nanoemulsions were successfully formulated and evaluated for morphology, zeta potential, droplet size, drug content, *in-vitro* drug release, pH, viscosity, % drug entrapment and refractive index.

From the FT-IR spectra it is concluded that, there is no interaction between the drug and excipients as the characteristic peaks of caffeic acid remained in the formulation too. The micromeritic properties were within the limits. The % drug entrapment ranged 92.70 ± 0.07. The *in-vitro* drug release was more than 90% for formulation in 0.1N HCl and phosphate buffer in 35 minutes. Hence, caffeic acid nanoemulsions provide better drug solubility and improved oral bioavailability that might be targeted to certain extent.

In addition to this, it also possess pharmacological activities like anti-proliferative, antioxidant, anti-microbial, anti-inflammatory, etc. that contribute its focused therapeutic potential.

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