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FORMULATION AND *IN-VITRO* CHARACTERIZATION OF 5-FLUOROURACIL AND FLAVONOID DUAL LIPID DRUG CONJUGATES LOADED SELF NANOMULSIFYING DRUG DELIVERY SYSTEM FOR CANCER TARGETING

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Keywords:

Chemotherapy, Multidrug resistance, 5-Fluorouracil lipid curcumin conjugates, Nanoemulsion

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ABSTRACT: Objective: The Failure in chemotherapy is mainly because of the resistance of the chemotherapeutic drugs towards the neoplastic cells. The main reason behind this study was to develop a novel combination of 5-Fluorouracil (5-FU) lipid curcumin conjugates for the treatment of cancer. **Methods:** In this study, the lipid group stearic acid and oleic acid was conjugated with the parent drug moiety 5-Fluorouracil to acquire lipophilicity. The next step involves the conjugation of curcumin with 5-Fluorouracil lipid to form a lipid dual drug conjugate. The characterization of conjugates followed the synthesis part by using FT-IR, DSC, and LCMS. The conjugates hence obtained were further carried for formulation aspect. After the successful completion of the formulation part, *in-vitro* studies and MTT Assay was performed. **Results:** The results depict the successful synthesis of the conjugates, and its characterization was successfully done. The conjugates are formulated into Self Nanomulsifying drug delivery system (SNEDDS). The results obtained from the MTT assay also shown a significant cytotoxic effect in both the conjugation. **Conclusion:** The *in-vitro* results suggest further studies for cleavage of the drug and the lipids in the conjugate and *in-vivo* animal studies to demonstrate the anticancer efficacy.

INTRODUCTION: Cancer is the uncontrolled growth of cells in our body. There are more than 100 types of cancer. The cancer is named based on the site from which it originates. Based on the tumor stage and characteristics, present treatment methods comprise radiation therapy, chemotherapy, surgery or the combination of two or more methods.

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Because of the absence of the acutely defined symptoms during the initial stages of cancer, the patients are usually diagnosed in the IVth stage that is during the advanced stage mostly after the initiation of metastasis. The use of two or more treatment may lead to remarkable toxicity, which is not appropriate to be used for elderly patients or other commodities [1]. Therefore it is important to discover a new agent or formulate a novel drug delivery system with better efficacy and with adequate tolerance.

The recommended chemotherapeutic drug of choice for this purpose is 5-Fluorouracil (5-FU) though other antineoplastic drugs are also available.

5-FU is the first line drug used in the treatment of cancer [2]. Because of its inefficacy in the therapy, due to the poor selectivity and the other side effects such as mucositis, myelosuppression, emesis, nausea, and hand-foot syndrome the formulation of this drug is constantly facing various challenges. The drug even has poor biopharmaceutical characteristics such as rapid drug catabolism, poor absorption and shorter biological half-life (10-20min) that hampers its formulation aspects. For modifying these side effects various novel approaches are in need to be evolved [3]. Some of them include a chemical modification, conjugation with polymers, lipids, *etc*. the other approaches are mainly based on the use of the Nano carriers such as Nanoparticles, Micelles, liposomes, *etc*.

One of the major challenges facing by the researchers nowadays is drug resistance. Drug resistance is the lowering in the effectiveness of a drug such as an antineoplastic agent in the treatment of a disease or a condition [4]. The effect produced by the antineoplastic resistance challenges both the clinical care and driven research. When an organism acquires resistance to more than one drug, then it is termed as multi-drug resistance. The major reason for the resistance mediated in most of the cancer cells is P-glycoprotein (P-gp), P-gp act as a barrier in reducing the intercellular drug concentration by inhibiting the drug influx and exhibiting the efflux across the physiological membrane of the cancer cells [5].

Curcumin is a flavonoid reported to exhibit anticancer property [6]. Curcumin reduces the side effects associated with cancer therapy such as toxicity and drug resistance. This drug even has the potential to destroy the cancer cells by deactivating the Notch signal pathway. It is also reported to have an additional use as it could reduce the Multidrug resistance (MDR) caused by P-gp [7]. It can also slow down cancer growth. So, in this regimen curcumin is used to produce a synergistic effect along with 5-FU. Conjugation of a drug with the lipid is to covalently modify the drugs with the lipids.

This strategy has demonstrated various benefits including enhancing targeting with tumor cells, improved oral bioavailability, reduces the toxic effect, and improved loading of the drug into delivery carriers. Lipid Drug Conjugate (LDC) also helps in the targeting of the drug to the lymphatic pathway, where the drug can avoid various challenges such as first-pass metabolism, poor absorption and gastrointestinal tract (GIT) irritation [8]. Most of the tumor cells need the lipids uptake for their rapid proliferation and to perform their metabolic activity. This lipid conjugate enhances the drug distribution to the cancerous cells and the release of the drug based on the cancer environment. So for this concept, both the lipid group and the chemical bond are required for the cancer targeting.

In our research, we mainly aimed at synthesizing a dual drug-lipid conjugate for the effective treatment to the cancer cells, the synthesis of the conjugates its characterization part followed by its formulation into nanoemulsion and its characterization. The *in-vitro* studies and MTT assay was also carried out.

MATERIALS AND METHODS:

Materials: 5-Fluorouracil (5-FU), formaldehyde, Dichloromethane (DCM), stearic acid, N, N^1 dicyclohexylcarbodiimide (DCC), Diethyl ether, N-hydroxysuccinimide (NHS), N, N^1dimethyll hydroxyl amine hydrochloride (DMH), Dimethyl sulfoxide (DMSO), was obtained from AVRS synthesis Pvt., Ltd, Curcumin was received as a gift sample from Himalaya Pvt., Ltd, and lauroglycon, Labrafil M and the other oils used are obtained from Gatteffosse India Pvt., Ltd.

Methods:

Synthesis of 5-Fluorouracil Lipid Curcumin Conjugates:

Synthesis of 5-Fluorouracil Lipid Drug Conjugates: Synthesis of the 5-fluorouracil and the lipid drug conjugates of stearic acid and oleic acid was performed *via* the two-step process. The first step involves the synthesis of *N*, *N*′-1, 3-bis (hydroxymethyl- 5-fluorouracil) which is intermediate and then in the next step only the 5-fluorouracil lipid conjugates was obtained [3].

In the first step about 10 mmol of 5-FU, 10mmol of formaldehyde and 25 mmol of distilled water was taken in a round bottom flask and was immersed in an oil bath at 60 º C and kept under agitation for 6 h. The product *N*, *N*′-1,3-bis(hydroxymethyl-5-

fluorouracil) was then obtained by a rotary evaporation method.

The conjugation of stearic acid with *N*, *N*′-1,3-bis(hydroxymethyl-5-fluorouracil) was accessed by using facile esterification method. About 10mmol of stearic acid was taken in a round bottom flask with 1.6 g of *N*, *N*′-dicyclohexylcarbodiimide (DCC) and 0.5 g of *N*, *N*′-1, 3-bis(hydroxymethyl-5-fluorouracil) in dichloromethane with 4-dimethylaminopyridine as a catalyst. These mixtures were allowed to stir at room temperature for 48 h. *N*, *N*′-Dicyclohexylurea was removed by filtration. Finally, the product 5-FU stearic acid was obtained by precipitation with diethyl ether followed by purifying with acetone for three times [9]. The conjugation of 5-FU oleic acid conjugates was also done by following the same procedure mentioned above by replacing the stearic acid with oleic acid. Enzymatic conjugation of 5-Fluorouracil lipid Curcumin conjugates.

Curcumin (0.4 g) is esterified with 5-fluorouracil and stearic acid conjugate (0.5 g) by enzymatic esterification method by using the enzyme lipase (0.2 g). This enzymatic reaction was run in a magnetic stirrer at 120 rpm and 45 ºC for 3 days. The product is purified with acetone 3 times [10, 11]. The conjugation of curcumin 5-FU oleic acid was done by following the same procedure by replacing stearic acid with oleic acid.

Characterization of the Conjugates:

FT-IR Spectra: FTIR spectra of the compounds were evaluated with the help of Spectrum RX 1 FTIR spectrometer (Shimadzu FTIR 8400 S, Japan). A disk of Potassium bromide (KBr) with the samples was prepared at a weight ratio of 4:1 of KBr, respectively. Peaks were acquired between 4000 cm^{-1} and 400 cm^{-1} and represented as the FTIR spectra [12].

DSC: DSC was used to find out the state of the synthesized compounds. DSC thermograms were performed in an automatic thermal analyzer system. Samples were weighed and placed in standard aluminum pans, sealed and heated between 20-310 ºC. An empty pan, sealed in the same way, was used as a reference [13].

LCMS: Quantification of the synthesized compounds was performed with an LC-MS system by using a dual ESI interface in positive ionization mode. The mass spectrometer was linked to a liquid chromatography system. The samples are being dissolved in DMSO and were analyzed. The LCMS was analyzed for 5-Fluorouracil Lipid Conjugates [10].

Formulation Approach:

Solubility Studies: Solubility of the conjugates was bent with different oils, along with the surfactants and co-surfactants. An excess amount of the conjugates was mixed with 2 ml of the solvents in a 10 ml volumetric flask and kept at 37 ºC at 100 rpm in an isothermal shaker. After 72 h the drug solvent mixture was centrifuged at 3000 rpm for 15 min and left undisturbed for another 72 h. The supernatant obtained was filtered through a 0.45 µm membrane filter and then diluted by using methanol. The number of conjugates solubilised was analysed using UV-VIS spectroscopy at 549 nm using methanol as the blank [14].

Preparation of Nanoemulsion Formulation: 40 mg of the conjugates was scaled up and dissolved in Smix (kolliphor and ethanol- surfactant and Co-Surfactant mixture) and sonicated for 2min followed by the addition of the oil mixture (Lauroglycol + labrafil M 2:1). This mixture was then triturated with the specified weight of water to obtain its homogeneity. For the effective entrapment of the drug in the selected formulae; the physical state and the transparency of the formulation was checked [15].

Thermodynamic Stability Studies: After the preparation of the formulation, it was subjected to various thermodynamic stability studies.

Heating-Cooling Cycle: The formulated nanoemulsion was dealt with six cooling and heating cycles between 4 ºC to 45 ºC storing in each temperature at for 72 h and observed for phase separation and precipitation.

Centrifugation: The formula that passes the cooling and heating cycle was subjected to centrifuge at 4000 rpm for 30 min and analyzed for phase separation and precipitation studies.

Freeze-Thaw Cycle: The formula that passes centrifugation test was subjected to undergo freeze-thaw study, where the formulae are kept in a deep

freezer between the temperature ranges from -21 ºC to +25 ºC for 48 h and visualized for phase separation and precipitation.

Percentage Transmittance: The conjugates were reconstituted with 50 ml of distilled water, 0.1M HCl and 6.8 pH phosphate buffer respectively and resulting nanoemulsion was visualized for any turbidity. The percentage transmittance was then measured using UV-VIS spectroscopy at 549 nm [16].

The confirmation of the Bond after the Formulation: After the formulation part, the conformation of the bonds after the formulation whether any stereotypical or the structural changes in the conjugates are determined by using FT-IR method. FT-IR spectra of the compounds were performed using a Spectrum RX 1 FTIR spectrometer (Shimadzu FTIR8400 S, Japan). Potassium bromide (KBr) disks containing the material of interest were prepared at a weight ratio of 4:1 of KBr, respectively.

The peaks were found in the range between 4000cm^{-1} and 400cm^{-1} and presented as the FT-IR spectra. Then the characteristic changes were evaluated by comparison between the conjugates before and after the formulation [12].

Characterization of Nanoemulsion: Particle size distribution, zeta potential, and polydispersity index (PDI) of the NE was determined by dynamic light scattering (DLS) technique. The instrument used is the Malvern Zetasizer Nano, Series ZEN1002 (Malvern, UK) in cuvette DTS0012 with a 532 nm green laser and a scattering angle of 173 °C [17].

***In-vitro* Drug Release Study:** [18] The release of 5-Fluorouracil lipid curcumin conjugates in SNEDDS systems was studied by evaluating the in vitro drug release by using Jerusalem Medium. The weighed amount of curcumin 5-fluorouracil lipid conjugates each in 50 ml of 7.4 pH Jerusalem medium was kept in a dialysis bag (12,000KDa) as a donor compartment and in receptor compartment 7.4 pH phosphate buffer was used.

The contents used for Jerusalem medium is represented in **Table 1**. The reaction mixture was incubated at 37 ºC and subjected to continuous shaking at 90 rpm, using a rotary shaker. At different time intervals, 1 ml of the reaction mixture was withdrawn [3]. The number of conjugates released was estimated using a UV-Visible Spectrophotometer at 549 nm.

TABLE 1: CONTENTS USED IN JERUSALEM MEDIUM

Contents	Quantity
Pancreatin	1.5 mg
Tributyrin	2.4 mg
Taurocholic acid	15 mg
Bile acid	2.8 mg
Phosphatidylcholine	40 mg
Calcium	2.4 mg
Tris-maleate	4 mg
Final volume	50 ml

Cell Line Studies: [19] The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The cleavage of MTT to a blue formazan derivative by living cells is an effective principle on which the assay is based.

RESULTS AND DISCUSSION:

Synthesis of 5-Fluorouracil Stearic Acid Curcumin Conjugates: After completion of the first step, the percentage yield of the product *N*, *N′*-1, 3- bis (hydroxymethyl- 5- fluorouracil) was obtained to be 77%. Then this is followed by the next step in the synthesis where the percentage yield of 5-FU stearic acid conjugates and 5-FU oleic acid conjugates was found to be 82% and 75% respectively.

After the third step, the synthesized conjugate of curcumin 5-fluorouracil stearic acid conjugate and curcumin 5-fluorouracil and oleic acid conjugate had a yield of 70% and 62%. The synthesis part is demonstrated in **Fig. 1**.

The facile esterification reaction obtained 5-FU stearic acid and 5-FU oleic acid conjugates. For the fatty acid either the application of heat or an acid/base condition is needed. DCC used in this reaction as an activating/ dehydrating agent and are used for building amide and a peptide linkage. The conjugation of lipids (stearic acid and oleic acid) and the flavonoids (antioxidants) involves the mechanism of enzymatic esterification using the enzyme lipase.

Characterization of the Synthesized Conjugates: DSC analysis of 5-FU and formaldehyde in comparison with *N*, *N*′-1, 3-bis(hydroxymethyl-5-fluorouracil) confirms the formation of product and the removal of starting material **Fig. 2**.

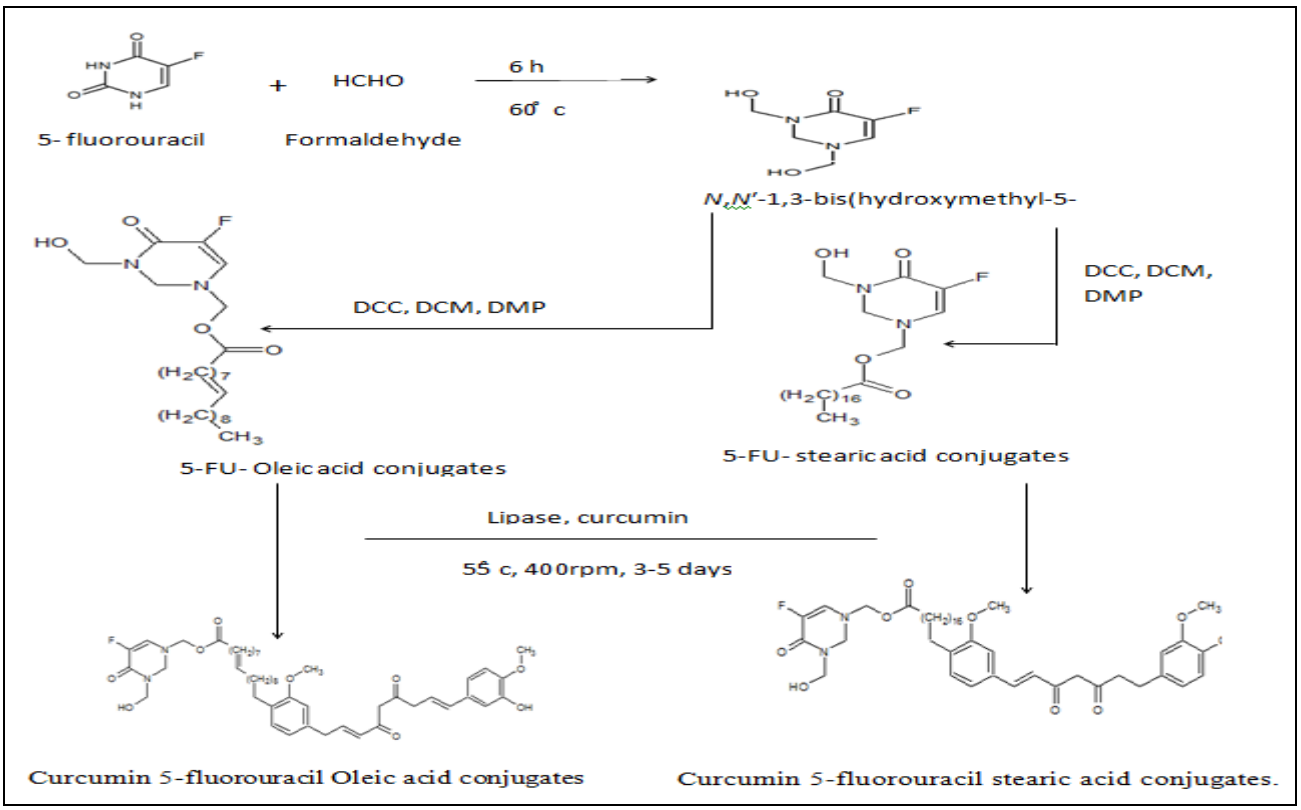

FIG. 1: SYNTHESIS PATH OF 5-FU LIPID CURCUMIN CONJUGATE

The melting point curves of 5-FU (melting point = 282 ºC) and formaldehyde (melting point - 92 ºC) were found to be absent. The *N*, *N*′-1, 3-bis (hydroxymethyl-5-fluorouracil) showed a Tg (glass transition temperature) at 68 ºC. The melting point curves of *N*, *N*′-1, 3-bis(hydroxymethyl-5-fluorouracil) (68 ºC) stearic acid and oleic acid (69.3 and 14 ºC respectively) were completely absent in the product. The 5-fluorouracil stearic acid conjugates and 5-fluorouracil oleic acid conjugates showed a Tg (glass transition temperature) at 206 ºC. The melting point curve of curcumin is found to be 183 ºC and the melting point of the curcumin 5- fluorouracil stearic acid conjugate and that of 5-Fluorouracil oleic acid curcumin conjugate are found to be 285 ºC and 281 ºC respectively this depicts that the products or the conjugates were formed.

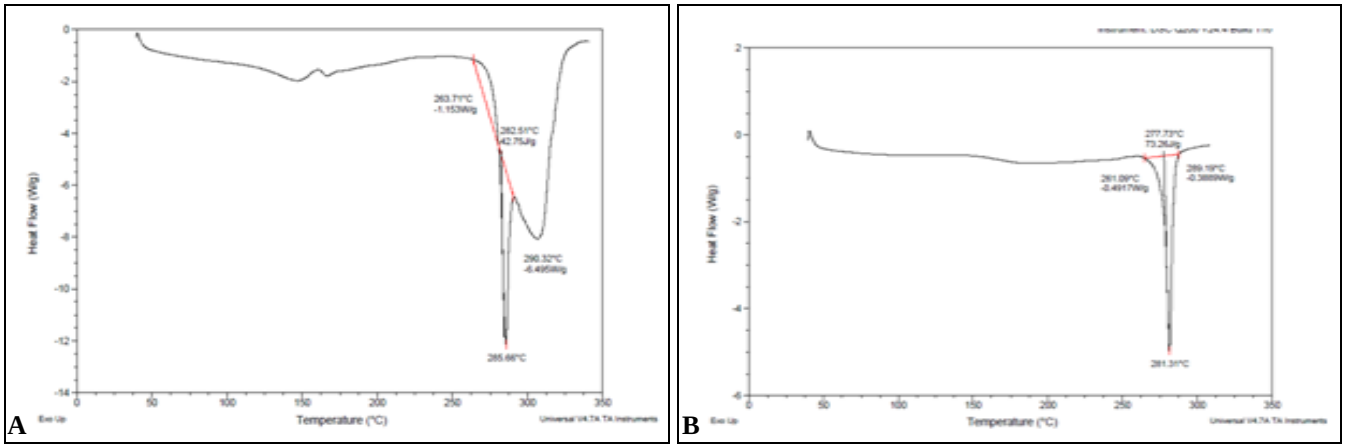

FIG. 2: A) DSC REPORT OF 5-FU STEARIC ACID CURCUMIN CONJUGATES. B) DSC REPORT OF 5-FU OLEIC ACID CURCUMIN CONJUGATES

LCMS reports were done to find out the second step, from the reports, obtained it confirms the formation of the 5-FU lipid conjugates **Fig. 3**. The products were formed by positive inotropic effect, and it shows ~90% of purity. From the molecular weight of the compound calculated it shows that the product formed shows similarity in its characterization. From the calculated molecular weight and the compound and the obtained weight, it depicts a purity of more than 90%.

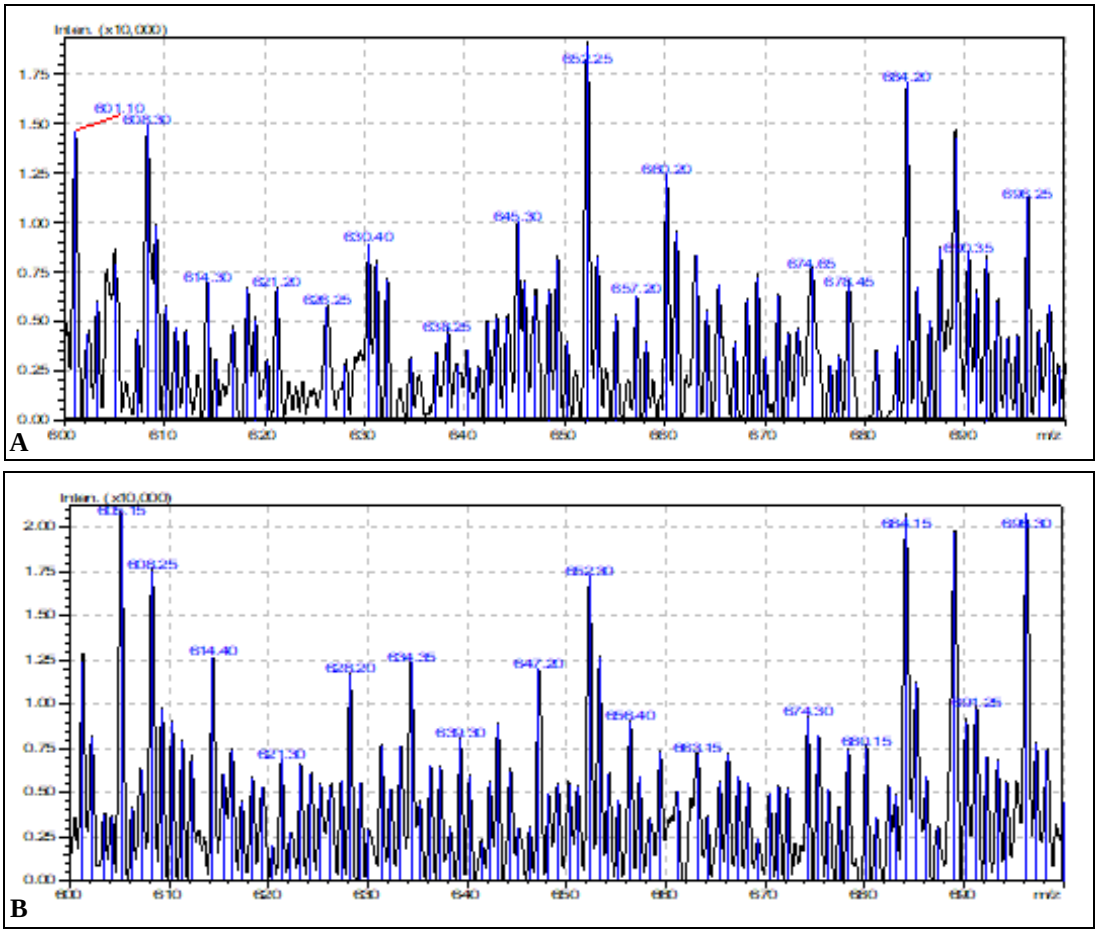

FIG. 3: LCMS REPORT OF A) 5-FU STEARIC ACID CONJUGATES B) 5-FU OLEIC ACID CONJUGATES

In FT-IR reports it depicts that in **Fig. 3** the presence of the N-H bond is missing which is present in the parent drug, *i.e.* 5-fluorouracil. From **Table 2** which represents the FT-IR data of the synthesized compounds, we can identify the association and the dissociation of the bonds in the synthesized compounds. The FT-IR report of the conjugates after its incorporation in oil is also reported in **Table 2**. The FT-IR report is given in **Fig. 4**.

TABLE 2: FT-IR DATA OF THE CONJUGATES

Functional group	Conjugate wavelength (stearic acid)	Conjugate wavelength (oleic acid)	Conjugates in oil wavelength (stearic acid)	Conjugates in oil wavelength (oleic acid)
C=O	1722.58	1709.58	1722.58	1710.25
C-N	1649.19	1653.19	1450	1654
C-H	1246.03	1243.03	1244.52	1244
C-O	1180.41	118.41	1180.14	118.20
C-F	1240	1243	1240	1240
=C-H	2943	2949	2943	2950
CH_2	2927	2955	2930	2954
O-H	3464	3358	3466	3359
C=H	2927	2955	2927	2954
CH_2.CH_3	1471	1436	1472	1436
C-CH	575	576	574	576

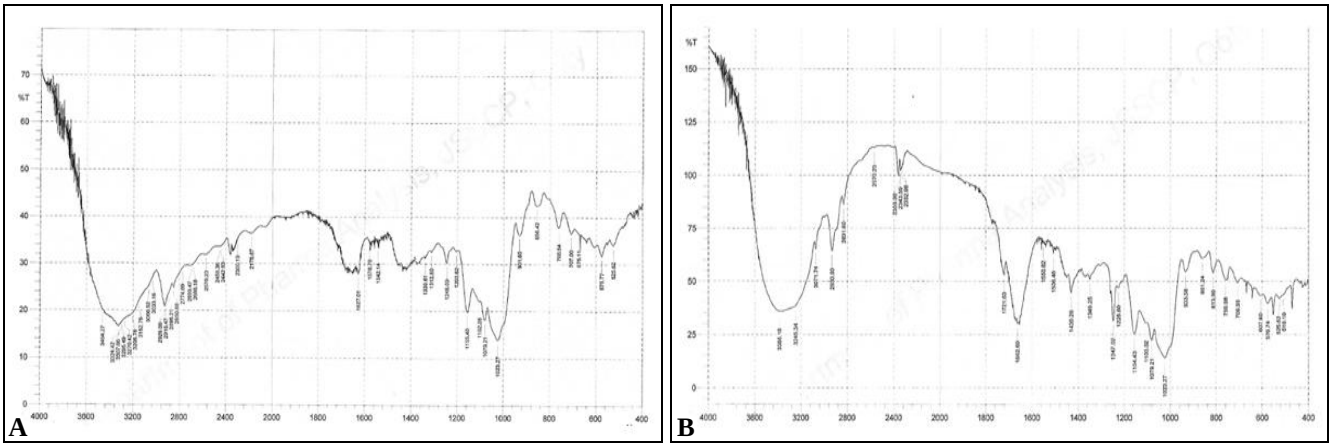

FIG. 4: FT-IR REPORTS OF A) 5-FU STEARIC ACID CURCUMIN CONJUGATES B) 5-FU OLEIC ACID CURCUMIN CONJUGATES

Solubility Studies: Solubility is an important criterion in the formulation of Nanoformulation. The oil phase in which the drug showed maximum solubility was selected for the purpose. From **Fig. 5**. It is evident that Lauroglycol + labrafil M (2:1) showed maximum solubility on 5-Fluorouracil stearic acid Curcumin conjugate 40 mg/mL and for 5-Fluorouracil stearic acid Curcumin in Lauroglycol + labrafil M (2:1) had shown a solubility of 5-Fluorouracil oleic acid Curcumin in Lauroglycol + labrafil M (2:1) was found with a solubility of 30 mg/ml. Hence, Lauroglycol + labrafil M (2:1) was selected for the formulation of NE. The increased solubility of the drug could be due to the more affinity towards the respective oil. Moreover the solvent properties of both the oils might result in a synergistic effect on the solubility of the conjugate. Various surfactant and cosurfactants mixtures were subjected for ease emulsification. From the studies, the mixture containing kolliphor EL and ethanol were selected for nanoemulsion formulation using spontaneous emulsification method. Pseudo-ternary phase diagrams were constructed (not shown) to select the optimized formulation. From the results 1:1 ratio of the same was selected for the formulation. The formulations passed the thermodynamic stability studies were subjected for further studies.

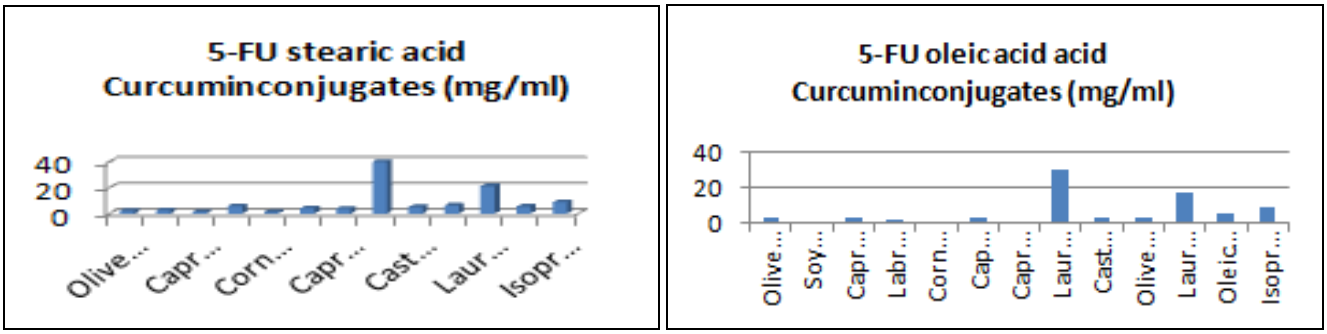

FIG. 5: SOLUBILITY STUDY OF CONJUGATES FORMED IN DIFFERENT OILS

Characterization: Particle size and polydispersity index of the formulated dual lipid drug conjugates were measured by photon correlation spectroscopy using a Malvern Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK). The Z-average particle size (dnm) of the nanodroplets formulation (5-Fluorouracil stearic acid Curcumin conjugates & 5-Fluorouracil Oleic acid Curcumin conjugate) was found to be 110.4 nm and 199.3 nm respectively. The capability of nanodroplets to make changes in the biodistribution and pharmacokinetics of drugs is important *in-vivo* therapeutic applications. In this aspect, the size and surface characteristics of nano-droplets are of major importance. Nanodroplets having a particle size of 100 nm are easily engulfed by Kupffer cells or other phagocytic cell populations that restrict their biodistribution.

The polydispersity index (PDI) is the measure of the size distribution of the nanoparticle formulation. PI was measured using Malvern Zetasizer. PDI values range from 0.01 to 1.000, *i.e.*

monodisperse to very broad particle size distribution. PDI values of all the formulations indicate that particle size distribution was unimodal. The optimized batch (5-Fluorouracil stearic acid Curcumin conjugates & 5-Fluorouracil Oleic acid Curcumin conjugate) having least particle size 110.4 nm had a PDI of 0.386 and with particle size 199.3 nm with PDI 0.326

Zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. The importance of zeta potential is that its value can be related to the stability of colloidal dispersions. The zeta potential generates the degree of repulsion between adjacent, similarly charged particles in the dispersion medium. For molecules and particles that are small enough, a high zeta potential will confer stability, *i.e.,* the solution or dispersion will resist aggregation. When the potential is low, attraction exceeds repulsion, and the dispersion will break and flocculate. The zeta potential of the conjugates was found to be -14.5 for 5-Fluorouracil stearic acid curcumin conjugate and -0.278 for 5-fluorouracil oleic acid conjugates.

***In-vitro* Drug Release Profile:** Before initiating the dissolution study it is necessary to study the fate of lipid in the GI tract. Hence, the formulation is subjected to lipid digestion, which is a normal phenomenon that occurs in the body when any food materials are taken orally. Being a lipid-based drug delivery system containing a dual drug conjugate, it is necessary to assess the release of the actual form from the lipid conjugates. For this purpose, a lipid digestion media with formula as given in **Table 1** was prepared and placed in diffusion tube (open at both ends) with one end covered with the dialysis membrane (molecular weight 12,000KDa). The formulation is then mixed with the lipid digestion media, and the whole diffusion tube was immersed in the phosphate buffer solution at pH 7.4.

The formulation undergoes digestion process, and cleavage of the conjugate take place leaving the free drug which diffuses or release through the dialysis membrane was analyzed at different time intervals for the quantitative amount of free drug getting released into the buffer solution. As the digestion takes place in the presence of a lipase, bile salt, and acid, the lipids basically which are triglycerides will break into monoglyceride, fatty acid, and water. This leads to exposure of lipid conjugate, *i.e.* stearic acid gets utilized by the media leaving behind the free drug.

The free drug diffuses out of the dialysis membrane which was analyzed from the dissolution data. The lag time for the releasing of the drug was found to be 3 min for the formulation containing curcumin stearic acid conjugate and curcumin oleic acid conjugates. Most of the drug after the digestion and cleavage gets released into the media within 60 to 80 min of exposure.

We could able to get the result without much change in the release behavior of both conjugates. Hence it can be made with both stearic acid and oleic acid for administration. Moreover, there is a difference in the solubility of both dual conjugates with stearic acid and oleic acid in oil from this point of view. From this point of view, conjugates formed with stearic acid could be taken for further characterization and study because the stearic acid conjugates showed better solubility than that formed by oleic acid. The percentage release of the drug from the media is given in **Table 3**.

TABLE 3: *IN-VITRO* DATA OF THE CONJUGATES

Time (min)	% drug release (5-FU stearic acid curcumin conjugates)	% drug release (5-FU oleic acid curcumin conjugates)
5 min	45.2	42.2
10 min	69.2	51.6
15 min	87.5	65.7
30 min	91.2	80.2
45 min	93.6	88.2
60 min	94.2	92.2
120 min	95.6	94.1

MTT Assay: The percentage growth inhibition was calculated using the following formula and concentration of drug or test samples needed to inhibit cell growth by 50% values were generated from the dose-response curves for each cell line MCF-7. The % growth inhibition is given in Equation (1).

% Growth inhibition = Mean OD of individual test group × 100 / Mean OD of control group ....(1)

The IC_{50} value of the curcumin 5- fluorouracil stearic acid conjugate is 67.60 µg/ml and that of curcumin 5-fluorouracil oleic acid conjugate is

85.83 µg/ml. From the results obtained from the MTT assay 5-fluorouracil oleic acid, curcumin conjugate is having more value than that of 5-Fluorouracil stearic acid curcumin conjugates hence 5-fluorouracil stearic acid curcumin conjugates which are having less cytotoxicity and can be formulated than compared to 5-fluorouracil oleic acid curcumin conjugate.

CONCLUSION: In our study, a novel formulation of 5-Fluorouracil stearic acid curcumin conjugates and 5-Fluorouracil oleic acid curcumin conjugates were synthesized, as a lipid dual drug conjugate for the first time. The conjugates were formulated by SNEDDS and characterized. *In-vitro* and MTT assay was carried out. Our report proves that the synthesized conjugates showed high efficacy and less toxicity than the parent drug. The use of the curcumin; a natural flavonoid is a good candidate for conjugation with 5-FU to enhance their specificity and efficacy towards cancer cells. Further detailed studies for anticancer efficacy are proposed in the next part of our research.

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CONFLICT OF INTEREST: None

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