ORIGINAL ARTICLE

Development, Anticancer Efficacy of Quercetin Nanoformulations Encapsulated in to Liposomes against Glioblostoma (U87) Cancer Cells

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ABSTRACT

The clinical potential of Quercetin is impeded due to its poor aqueous solubility, low bioavailability and lack of cancer specificity. Since convention approaches in the clinical development met failure, Nano dosage form was developed successfully to overcome the difficulties faced. Chitosan was used as a polymer in the Quercetin Nano formulation which was developed by the mechanical shaking method. As compared to the Quercetin, Developed Nano formulation was produced superior cytotoxicity in the glioblostoma cells(U87 cell). Developed Quercetin Nano formulation can have improved dissolution and it could able to cross the BBB and have and potential application on the cancer therapy. Moreover, it could enhance tumor localization and cancer specific therapy in the glioblostoma cells(U87 cell) by the induction of ROS mediated mitochondrial membrane depolarization, nuclear condensation and DNA fragmentation. Quercetin Nano formulation was characterized by zeta Potential, Particle size distribution, Entrapment efficiency, SEM Images, glioblostoma cells(U87 cell) studies and invitro drug release studies. The study provides a strong evidence for the use of the quercetin loaded liposome Nano formulation as an agent for brain targeting, chemotherapeutic and cytotoxic activity, therefore developed formulation will greatly improve the efficiency of future cancer therapies with cost-effective treatment.

KEYWORDS: Quercetin Quantum Dots; liposomes; glioblostoma cells(U87 cell); nano formulation; cytotoxicity

Introduction

Zinc Oxide Quantum dots (ZnO QDs) are the semiconductor nanocrystal which have extensive optical properties and owed quantum confinement effect [1]. An innovative optical, catalytic andelectrical application of the semiconductor causes development of semiconductor nanocrystals. These nanocrystals were used in various extensive applications from optoelectrical to bioimaging. Since its versatile nature of biological labeling, the drugs can leads to identify the site of action in targeting. ZnO (Zinc oxide) having wide band gap (3.37 eV) and their excitation was stable at room temperature. About 3.0–4.5 $\times 10^{-2}$mmol (2–3 g) of Zinc present in a human muscle, bone,skin and plasma which further involved in biological functions. Hence usage of ZnO in a formulation can be ecofriendly and suitable material for bioimaging [2].

Nanocarriers like liposomes have a versatile application in drug delivery through which the drug can be reaches the targeted site by overcoming limitations solubility and permeability [3-6]. Polymeric micelles of the liposomes are arranged with hydrophobic core that will increase the solubility of poorly water soluble drugs moreover the hydrophilic core will prevent the interactions between the core and other biological fluids. Nowadays, due to the development of the research area in “theranostics” for the imaging, diagnosis and treatment using MNPs (Multifunctional Nanoparticles)for personalized medicine are being developed [7-10]. Bottom up method was used for the preparation of liposomes. By reducing the size of the formulation, it is possible to increase solubility, optimum bioavailability and reduced toxicity of the drug substance.

Quercetin (3, 3’, 4’, 5, 7-pentahydroxylflavone) is compound that has well known potential benefits for cancer therapy as a free radical scavenging substance. Since poor solubility and bioavailability of Quercetin, it fails in clinical development. Previous belief on

the dietary Quercetin was without absorption Quercetin was excreted in the feces. But on the recent studies suggest Quercetin was absorbed in the GI Tract and it is metabolized in the various stages [11]. Quercetin in association with monoglucoside (Isoquercetin and Q4'G) in GI tract, when it is absorbed it will converted as a Quercetin aglycone part, further it will be hydrolyzed by the Lactose Phenizin Hydrolase (LPH) at the site of brush border. In other hand, Quercetin in the glycoside mixture contains hyperoside (such as glucose) or rutin (such as disaccharides/oligosaccharides) which absorbed into the large intestine by the enterobacterium [12]. It is metabolized through the various pathways and enzymes likely uridine-50 -diphosphateglucuronosyltransferase (UGT),phenol sulfotransferase (PST) and catechol-O-methyl transferase (COMT) which results in the formation of Quercetin metabolite such as Q30 GA (Quercetin 30-O-b-D-glucuronide), Q40 GA (Quercetin 40-O-b-D-glucuronide), Q3GA (Quercetin 3-O-b-Dglucuronide), 30 -O-methyl Quercetin 3-O-bD-glucuronide, 30 -O-methyl Quercetin 40 -O-b-D-glucuronide and Quercetin 30 -O-sulfate. At last there is no Quercetin aglycone is bioavailable [13]. Though Quercetin is converted into the metabolite, recent studies supported that some of the metabolite produces the beneficial actions. For example: Q3GA in free radical scavenging activity and Q30 GA and Q40 GA in inhibition of XOD and LOX [14]. During inflammation glucuronide metabolite releases flavonoid aglycones by enhanced b-glucuronidase activity. [15]Moreover the recent research and development suggests that conjugated quercetin is present in the blood circulation, and it will produce the action whenever it needs. [16] Because of this reason, Quercetin was administered in IV route. For targeted action, Quercetin needed cautious formulation development through which the drug can reach the targeted site. For brain Targeting the Quercetin can be administered either in oral or intranasal route. Based on this information, Quercetin was selected as a model drug to develop a Nano formulation by utilizing the novel technologies to overcome the above limitation.

Materials and methods

Preformulation studies

The drug with excipients which are used for the formulation development can be investigated in various parameters to estimate the stability and compatibility of drug with excipients to promote safe, efficacious and stable formulation with quality. Hence, the following studies done prior to the formulation development [17].

Characterization of drug

Appearance of the drug

The drug sample procures were investigating for its appearance, color and results were recorded.

Determination of purity

Purity was determined by the melting point. Melting point are unique for individual chemicals. Impurities can be identified by broadening or changing the melting point. Melting point determination of the obtained Quercetin sample was done by open capillary method. The equipment was calibrated before performing the analysis. Quercetin was compacted into capillary tubes (6 mm long, 1 mm diameter). The capillaries were introduced vertically and initial heat can be kept as 10 °C min and gradually increased. The Quercetin was performed in triplicate. Melting point of the drug can be determined and compared with reference which is cited in Indian pharmacopeia 2007.

Determination of λ_{max} for quercetin

10 mg of quercetin was dissolved in 100 ml 0.1N HCl (pH 1.2) to prepare 0.1 mg/ml stock solution (Solution A). From Solution A, dilute (10 µg/ml) solution was assembled and scanned by UV-VIS spectrophotometer at the range of 200-400 nm to obtain the λ max of Quercetin.

Preparation of standard curve

Calibration curve of quercetin was done in 0.1 N HCl (pH 1.2). A serial dilution of the drug from 10 µg/ml to 80 µg/ml was prepared. The prepared dilutions were analysed in UV Vis spectrophotometrically at λ_{max}. The absorbance vs. concentration was plotted, and beer's range was determined. The data's were analyzed in triplicate, and standard deviation was represented.

Fourier Transform Infra- Red Spectroscopy

KBr Pellet method was used to record FT - IR spectra in shimadzufourier transform infra-red spectrometer. Sample was prepared in the ratio of 1: 100 of drug and KBr. All the samples were scanned over a frequency range of 4000-400 cm^{-1} [18].

Formulation of quercetin quantum dots

Preparation of zinc oxide (ZnO) quantum dots

Zinc oxide (ZnO) quantum dots were prepared by Bottom Up method. Zinc chloride was dissolved in nitric acid (HNO_3) (Solution A). Methanol was added to Solution A and dried at a suitable temperature. Excess methanol can be added to this mixture to ensure the complete dissolve of $ZnCl_2$ (Solution B). Sodium Hydroxide was added to Solution B with vigorous stirring to precipitate ZnO. The temperature was maintained for a specific period of time to

the solution becomes translucent. After that, the quantum dots started to precipitate and the solution become turbid and then cool the solution. Solution was kept for Centrifugation and the solution was filtered, dried and stored.

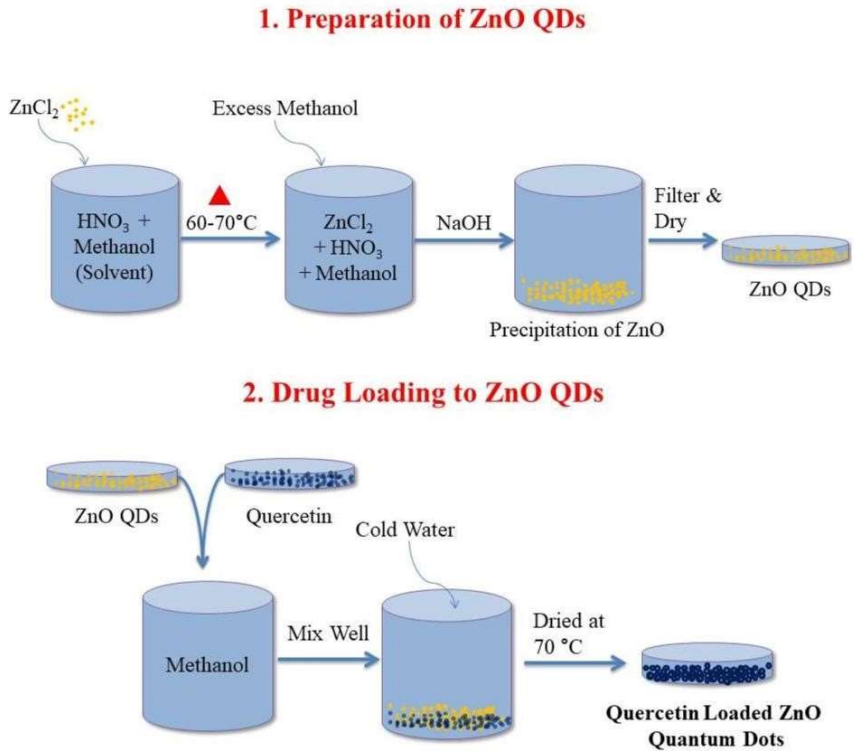

Fig.1 Preparation of Zn QDs and drug loading to Zn QDs

Drug loading on ZnO quantum dots

Initially, QDs were dispersed in methanol (Solution C). Quercetin was completely dissolved in Solution C and mixed well (Solution D). Cold water was added slowly to Solution D. The role of cold water was used to coat quercetin on the QDs. Quercetin came out and loaded up on the uncoated QDs. This drug loaded QDs were further dried in the oven at 70 °C.

Polymer coating on drug loaded quantum dots

Polymeric solution was prepared by dissolving the Chitosan in chloroform. The drug loaded QDs were weighed and then dispersed in the polymeric solution. This mixture was kept in undisturbed condition for 15 min and then stirred well. This mixture was filtered using Whatman filter paper. Polymer-coated QDs were dried at room temperature for overnight.

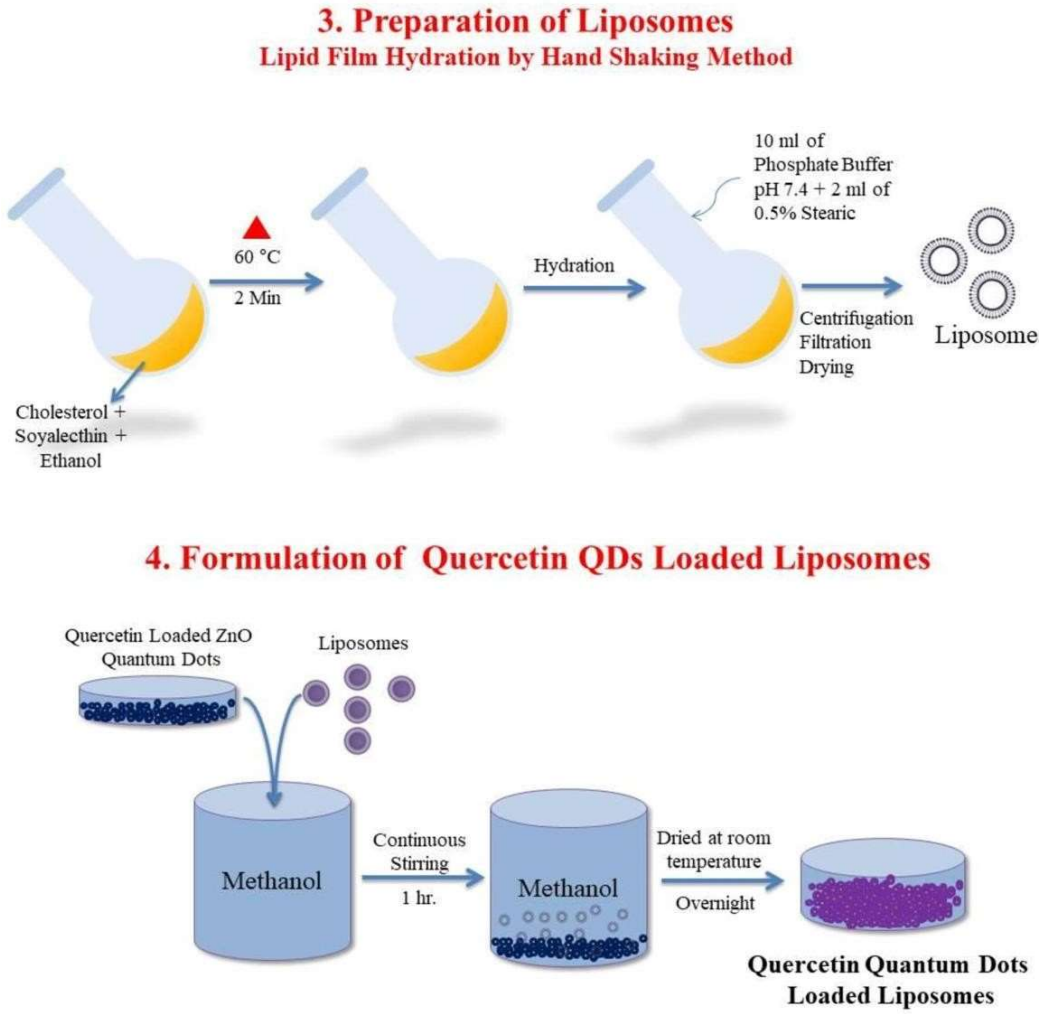

Fig.2 Preparation of Liposomes and formulation of Quercetin QDs loaded Liposomes.

Formulation of Quercetin Quantum Dots

Quercetinin nanoformulation was prepared by mechanical shaking method using polymer such as chitosan in various proportions. The ratio of quercetin formulation are representated in Table 1.

Table: 1 Composition of drug loaded using different ratio of polymer.

S.No	Formulation Code	Drug (g)	Polymer (g)
1.	F1	1	0.5
2.	F2	1	1.0
3.	F3	1	1.5
4	F4	1	2.0
5	F5	1	2.5

Formulation of liposomes

Lipid film hydration by hand shaking method was used to prepare the liposomes. Cholestrol and Soya Lecithin was weighed accurately and dissolved in an ethanol (Solution E) at 60 °C and stirred for two minutes. Liposomes were prepared by hydrating the solution E with continuous stirring by adding 10mlphosphate buffer pH 7.4 and added 2ml of 0.5% stearic acid for about 1 h. The obtained liposomes were stored in a hermetically sealed container.

Formulation of liposome loaded quercetin quantum dots

The liposome loaded Quercetin QDs was prepared by dissolving Quercetin QDs in Methanol initially, later the obtained liposomes was added in a same solvent with continuous stirring for few hours. Then the mixture kept at room temperature at overnight.

EVALUATION OF LIPOSOME LOADED QUERCETIN QUANTUMN DOTS

Particle Size analysis

Microtac blue wave particle size analyser was used for the particle size analysis of Quercetin QDs. Using this method, confirmation of the nano scaled particles can be made.

Zeta Potential Analysis

Zeta potential analysis of liposome loadedQuercetin quantumdots formulation was carried out using Malvern zeta seizer (Malvern instruments). The Zeta potential of the colloidal system was measured through the electrophobic mobility by which the particle charge can be confirmed. The Sample was prepared using the deionized water and NaCl was used to adjust conductivity [19].

Drug Content Determination

UV Vis Spectroscopic Method was used for the quantification of Quercetin QDs. The prepared quercetin quantum dot was equivalent to 10 mg of quercetinweighed accurately and dissolved in 10 ml methanol. Later, the stock solutions were diluted with distilled water and analysed by UV Vis spectroscopy at 272 nm. The drug content was determined by using the equation (1)

$$Drug\ Content = \frac{\text{Abs.of sample X Wt of standard X Total weight}}{\text{Abs.of standard X Wt of sample}} \text{X } 100 \quad \text{------Equation (1)}$$

Determination of Entrapment Efficiency (EE) of quercetin quantum dot

10 ml of quercetinquantm dot was centrifuged for 20 min at 5,000 rpm. The supernatant solution was filtered and separated. 1 ml of this filtrate was diluted with water and the absorbance was measured in UV Vis spectrophotometer at 272 nm using water as blank. The quantity of free drug in the formulations was measured, and the entrapment efficiency is then calculated by using equation (2).

$$EE\ \% = \frac{Total\ Drug\ taken - Free\ drug}{\text{Total Drug taken}} \text{X } 100 \quad \text{----------Equation 2}$$

Saturation Solubility Studies

10 mg ofunprocessed quercetin and quercetin formulation equivalent to 10 mg of quercetin were weighed and separately introduced into 25 ml stopper conical flask containing 10 ml distilled water. Both the flask was kept in a rotary shaker at 37°C for 24 h. After that the solution was collected and quantified in UV Vis Spectroscopy at 272 nm.

***In-Vitro* Drug Release Studies**

The *in- vitro* release of pure Quercetin and Quercetin loaded Nanoformulation were carried out in USP dissolution test apparatus IIusing 900 ml of HCl Buffer (pH 1.2) as well as phosphate buffer (pH 7.4) as a dissolution medium at 50 rpm and temperature maintained at 37.0 ± 0.2 °C. 10 mg ofQuercetin and Quercetin loaded Nanoformulation equivalent to 10 mg of Quercetin were used as a sample. The dissolution samples were taken at a specific time intervals. The sample was filtered and analysed at 272 nm using UV spectrophotometer. Finally the dissolution of Quercetin was compared with Quercetin loaded Nanoformulation.

Kinetics and mechanism of drug release

Release pattern of the Drug can be studied in various numbers of kinetic models. Zero, First, Higuchi and Peppa's are the model dependent methods that were used to elucidate the release rate [20]. *In vitro* drug release data was obtained for all the formulation and the following data can be obtained to describe the pattern of release.

Table : 2 **Kinetics models and estimation of drug releasemechanism**

Model	**X axis**	**Y axis**	**Confirmation of release pattern by linearity**
Zero order kinetic model	Cumulative % drug released	Time	Confirmation of Release pattern by Regression value = 1
First order kinetic model	Log cumulative percentage of drug remaining	Time	
Higuchi's model	Cumulative percentage of drug released	Square root of Time	
Korsmeyer equation/ Peppa's model	Log cumulative percent drug released	log Time	

a) Zero Order Release Kinetics

Zero Order Release Kinetics represent that the drug dissolution from dosage forms with not disaggregate and release the drug slowly. The equation (3) used for the Zero Order Release Kinetics

$Q = K_0 t$ equation (3)

Where, Q = fraction of drug released at time‘t’

K_0 = Zero order release rate constant

b) First order release kinetics

The first order release kinetics represents concentration dependent. equation (4)was used to study the First order release kinetics

$\ln (1-Q) = - K_1 t$---------- Equation (4)

Where, Q = Fraction of drug released at time‘t’

K_1 = First order release rate constant

c) Higuchi equation

The kinetic model through which the drug releases from the matrix system was first proposed by the Higuchi. Equation (5) was used to find the Higuchi order of kinetics.

$Q = K_2 t^{1/2}$

Where K_2 = release rate constant

d) Korsmeyer equation/ Peppa'smodel

Another familier model used to find the mechanism of drug release and the drug release behavior from polymeric systems. The equation (6) was used to describe Korsmeyer/Peppa'slaw.

$$Mt /Ma = Kt^{n}$$

Where,

Mt /Ma = the fraction of drug released at T.

K= Constant incorporating the structural and geometrical characteristics of the

Drug/polymer system.

N=Diffusion exponent related to the mechanism of the release.

Equation (6) can be simplified by applying log on both sides; we get equation (7)

$$LogMt /Ma = LogK + nLogt \text{--------- equation (7)}$$

When the data is plotted as log of drug released versus log T, yields a straight line with a slope equal to "n" and the "K" can be obtained from y intercept.

Table.3: Mechanism of Drug Release as per Korsmeyer Equation/ Peppa'sModel

S. No.	Drug release	n Value
1	Fickianrelease	0.45
2	Non– Fickian/anomalous release	0.45<n <0.89
3	Class II transport	n>0.89 <1
4	Super case transport	n>1

Scanning Electron Microscopy (SEM)

Surface methodology can be studied using microphotographs under different magnification in SEM. The scanning electron microscopy of liposome loaded Quercetin QDs was carried out to confirm the Nano sized formulation. In a high vacuum evaporator, using a gold sputter module the sample was sprinkled on an adhesive tape then stuck on an aluminium stub and then coated with 10 Å thickness of platinum under an argon atmosphere. The stubs containing the coated sample were placed in SEM [21].

Fourier Transform infra-red spectroscopy (FT-IR) analysis

Quercetin QDs interaction and stability of drug during microencapsulation process was analysed by the Fourier transform infra-red analysis. FT-IR spectrophotometer (Shimadzu 8400 S, Japan) was used for analysis of various samples. About 2-3 mg of samples were mixed with dried potassium bromide of equal weight and compressed to form a KBr disc. Fourier transform infra-red spectrum of purequercetin, soya lecithin, cholesterol, chitosan, and liposome loaded quercetin quantum dot were scanned over a frequency range 4000-400 cm^{-1}.

Thermal analysis

Differential scanning colorimetric (DSC) analysis (Toledo-DSC II instrument) was used to study the thermal properties of Quercetin QDs. Internal structural changes of pure Quercetin, polymer and the Quercetin QDs can be characterized. About 5 mg of samples were taken in an aluminium pan and kept in an instrument. The sample was then heated from 20 °C to 200 °C at a heating rate of 10 °C/min under a stream of nitrogen at flow rate of 50 ml/min.Enthalpy changes (ΔH) were calculated peak to study the polymeric changes in the formulations [22].

Powder X-Ray Diffraction Pattern

Physical nature of the substance can be studied by using X-ray powder diffractometry. The physical form of Quercetin was found to be crystalline which was dispersed in the QDs was subjected to X-ray powder diffractometry. Instrument was operated under the fixed parameters ofCu as target filter having a voltage/current of 40 KV/40 Ma at a scan speed of 1°/min.The samples will be analysed at a 20 angle range of 5-70° [23].

Stability Study

Stability study of the developed nanoformulation was conducted as per ICH guidelines. The optimized formulation was wrapped using laminated aluminium foils and kept in the accelerated stability chamber at different storage condition like room temperature, refrigerated temperature and elevated temperature as well as humidity condition of 40 ± 2 °C/75 ± 5 %. Stability of the nanoformulation was studied by comparing the stability of the formulation before and after the study through for the drug content, *in-vitro* drug release and particle size analysis.

***Invitro* cell line study**

Glioblostoma Cells [24-26] seeded at a density of $2.5*10^4$ cells 100 ul^{-1} in 96-well plates.The cells will be allowed to adhere for 24 h and then will treat with drugs suspended in culture medium at concentrationsof 0, 50, 100, 500, 1000, and 2000 $\mu g/ml^{-1}$ for 72 h in an incubator (37 °C, 5% CO_2/95% air atmosphere). Afterincubation, solutions removed and 50 µl of a $2mg/ml^{-1}$ solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide(MTT) in PBS were added to each well, and incubationwill be continued for a further 4 h at 37°C.Absorbance will be measured at 540nm using a microplate reader. The percentage of viable cells will be calculated using the equation (8)

$$\text{Viability (\% of control)} = \frac{(\text{A sample} - \text{A blank})}{(\text{A control} - \text{A blank})} \text{x}100 \text{ ---------} (8)$$

RESULTS AND DISCUSSION

PREFORMULATION STUDY

Physical observation

Quercetin was appeared in yellow crystalline powder. The taste and odour of pure drug was found to be bitter. The description of the substance is appropriate to the specifications informed by the manufacturer.

Solubility studies

Quercetin is soluble in organic solvents such as ethanol, DMSO, and dimethyl formamide (DMF). The solubility of quercetin in these solvents is approximately 2 mg/ml in ethanol and 30 mg/ml in DMSO and DMF. Quercetin is sparingly soluble in aqueous buffers.

Melting point determination

The melting point of quercetin was determined by using capillary method, and it was found to be 315-316 °C (standard 315 °C), the result which complies with the report value.The results found were compared to those obtained with the DSC technique.

Ultra-violet (UV) absorption spectra

The quercetin was analyzed by spectrophotometrically in between200 nm – 400 nm. The maximum absorbance (λ_{max}) was found for quercetin at 272 nm which is comply with the standard used for quantitative analysis. From Figure1 it was observed that the quercetin UV spectrum was mentioned in 0.1N HCl buffer.

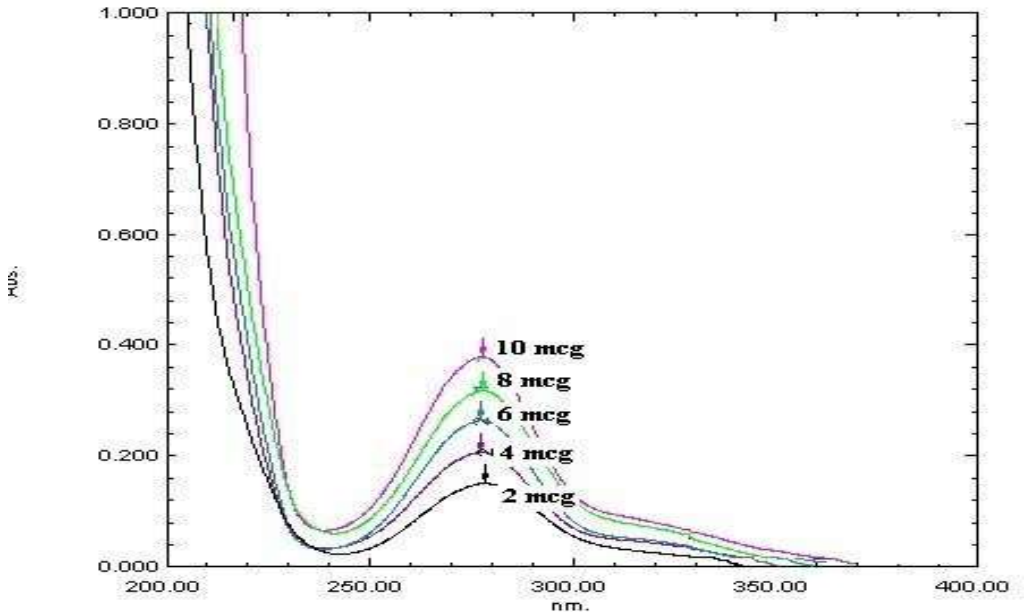

Fig.3 UV Spectrum of quercetin in 0.1HCL

PREPARATION OF STANDARD CURVE OF QUERCETIN

Standard calibration curve of Quercetin in 0.1 N HCl

The standard curve was prepared by using various concentration of quercetin versus absorbance at 272 nm. The calibration curve was found linear at different concentration at range in 10 – 100 µg/ml. From the below data (Table.3) it was observed that the drug obeys beer's law in concentration range of 10 - 100 µg/ml in 0.1 N HCl. The slope is 0.00155, the correlation coefficient is 0.999 it follows fit curve since r^2 is below

Table:4 Standard curve of quercetin in 0.1N HCl

S.No	Concentration in µg/ml	Absorbance at 272 nm
1.	10	0.017
2.	20	0.032
3.	30	0.049
4.	40	0.067
5.	50	0.081
6.	60	0.096
7.	70	0.112
8.	80	0.127
9.	90	0.142
10.	100	0.156

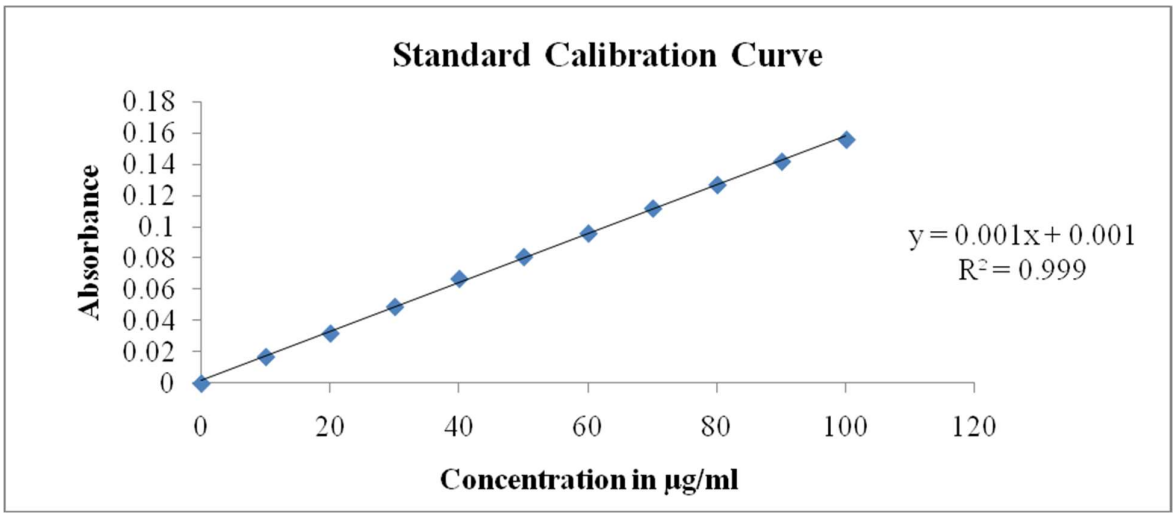

Figure.4 Standard curve of Quercetin in 0.1N HCl

Drug Polymer Compatibility study

The FT-IR spectroscopy was used to study the possible interaction between pure Quercetin, chitosan, soya lecithin and cholesterol. The drug polymer compatibility study was shown in the figure 3,4,5,6 and 7, table 2. The characteristic peaks for Quercetin can be observed at a wave numbers showed the presence of numbers ~3406.05, 3319.26cm^{-1} (Free O – H stretching alcohols ad phenols vibrations), ~2902.67-2540.08cm^{-1} (O – H stretching carboxylic acid vibrations), ~2902.67cm^{-1} (CH stretching alkane), ~1731.96 cm^{-1} (C=O stretching carbonyl) and ~1666.38 cm^{-1}(C=C alkanes). ~1521.73(C=C stretching heterocyclic aromatic. ~1452.30 (C-H bending alkanes). ~ 1319.22-1014.49(C-O Stretching Alcohol, Carboxylic acid). ~941.20-657.68(=C-H Bending Alkenes). Similar peak were seen in physical mixture of Quercetin and polymers. There was no discriminable shift/disappearance/appearance of peaks in combined spectra that indicated good drug – polymer compatibility and no chemical interaction between Quercetin and polymers. Hence, the selected polymers were found suitable for development of the

nanoformulation.

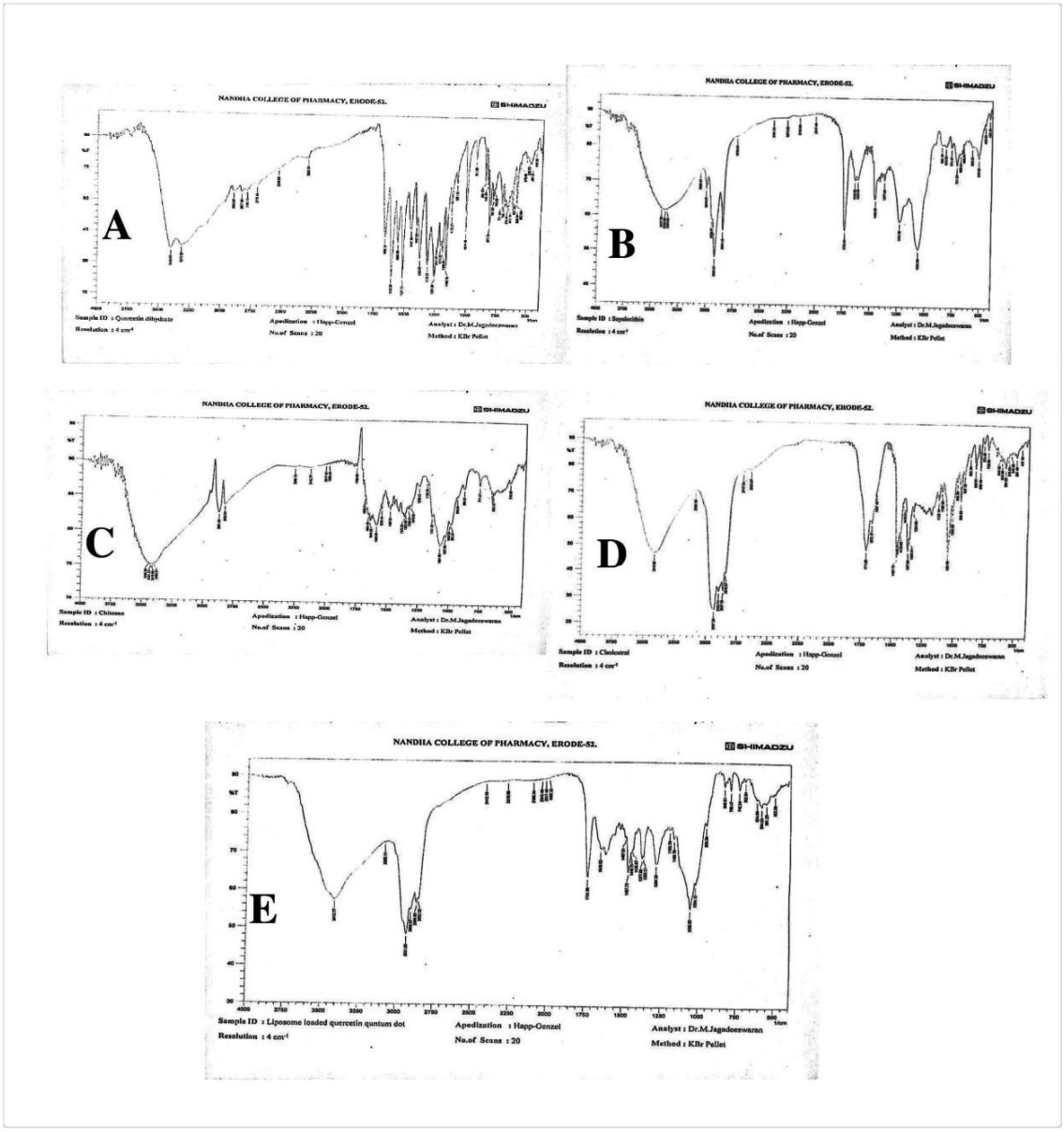

Fig.5 FT IR Spectrum for A. quercetin; B. Soya lecithin; C. Chitosan; D. cholesterol; E. Quercetin QDs loaded liposomes.

Table:5 Interpretation of IR spectrum of quercetin, chitosan, Soya lecithin, cholesterol, quercetin loaded liposomes formulation

Transition	IR Range (cm^{-1})	Absorption wave number (cm^{-1})				
		Quercetin	Chitosan	Soya lecithin	Cholesterol	Quercetin loaded Liposome formulation
O-H Stretching Alcohols, phenols	3500 - 3200	3406.05, 3319.26	3456.20-3400.27	3417.63-3363.62	3419.56	3413.77
O-H Stretching Carboxylic acid	3300 - 2500	2902.67-2540.08	2881.45, 2833.24	3066.61-2732.94	3099.39-2653.87	3080.11-2852.52
C-H Stretching Alkane	3000 - 2850	2902.67	2881.45	2956.67-2854.45	2937.38-2867.95	2931.60-2852.52
C=O Stretching Carbonyl	1760 - 1665	1731.96	1695.31	1735.81	1714.60, 1672.17	1731.96
-C=C- Stretching Alkenes	1680 - 1640	1666.38	1664.45	1649.02	1672.17	1649.02
C=C	1550 –	1521.73	1487.01	-	-	1487.01

Stretching Heterocyclic aromatic	1475					
C-H Bending Alkanes	1470 - 1450	1452.30	-	1458.08	1467.73	1467.73
C-H Rocking Alkanes	1370 - 1350	-	1363.58	1377.08	1365.51	1365.51
C-O Stretching Alcohol, Carboxylic acid	1320 - 1000	1319.22-1014.49	1319.22-1004.84	1234.36, 1066.56	1132.14-1022.20	1284.50-1024.13
=C-H Bending Alkenes	1000 - 650	941.20-657.68	993.27-655.75	860.19-659.61	985.56-738.69	958.56-702.04

9.2 EVALUATION OF NANOFORMULATION

9.2.1 Saturation solubility studies

Solubility determination in various pH media is an imperative in drug development because it gives the complete idea of drug behavior in various pH media. quercetin is practically insoluble in water. Based on the solubility data, a good dissolution medium was developed which is essential for absorption of drugs. quercetin nanoformulation showed enhanced solubility (101.34 ± 0.56 mg/L in 0.1N HCl and 165.71 ± 0.61 mg/L in phosphate buffer).

It was observed that the solubility of prepared nanoformulation has been increase to 6.1 ± 0.2 folds in pH 1.2 and to 7.5 ± 0.4 folds in pH 7.4 due to the formation of stabilized nanosuspension. The solubility of prepared nano formulation in 0.1 N HCl (pH 1.2) and phosphate buffer (pH 7.4) are represented in Table.

Table. 6 Saturation solubility studies of pure drug and nanoformulation.

S.No	Formulation Code	Absorbance at 272 nm (mg/L)	
		PHOSPHATE BUFFER	0.1 N HCL BUFFER
1.	Pure	19.67 ± 0.04	25.45 ± 0.23
2.	F1	53.73±0.56	95.21±0.61
3.	F2	61.34 ± 0.17	74.71 ± 0.53
4.	F3	55.45±0.16	72.10±0.61
5	F4	58.10±0.91	64.17±0.81
6	F5	48.28±0.61	61.61±0.51

Each value represents the mean ± SD (n=3).

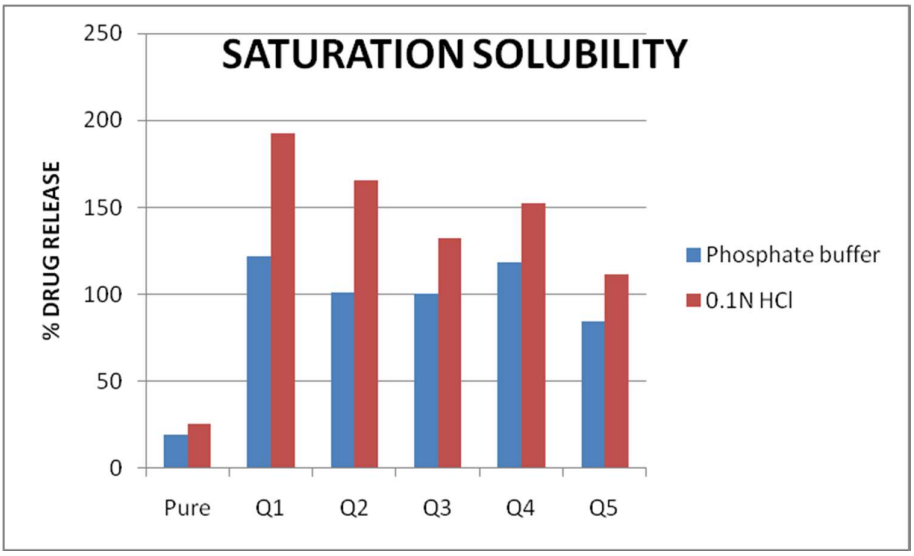

Fig.5 Saturation solubility studies of quercetin and quercetin nanoformulation

Drug content determination

The method used in this study appears to be reproducible for preparation of quercetin nanoformulation. The drug content of quercetin formulation results shows in table. The results showed that the formulated quercetin nanoformulation have shown the presence of high drug content with low standard deviation, and loss of drug was lower during preparation process. It indicates that the drug was uniformly dispersed in the powder formulation. Drug-loaded nanoformulation emulsified with chitosan can achieve higher drug content and cellular uptake, and thus higher therapeutic effects. From the formulation, F1 nanoformulation showed a high percentage of drugs loading which makes the delivery of drug clinically feasible.

Drug entrapment efficiency

The percentage drug entrapment efficiency of all the formulations was calculated and mentioned in table.. It is believed to occur because the surfactant decreases the interfacial tension between the polymer and aqueous phase. The quercetin entrapment efficiency of F1 (94.75 ± 0.62 %) was high when compared to other nano formulations. It might be due to the

presence of optimum polymer concentrations, comparing the remaining formulations. The concentrations of stabilizer used are the most effective factor on entrapment efficiency.

Table:7 Percentage of drug content & drug entrapment efficiency of quercetin nanoformulation

S.No	Formulations	Drug content	Drug entrapment efficiency (%)
1.	F1	95.49 ±0 .63	70.0 ± 0.23
2.	F2	96.61 ±0.32	82.75 ± 0.62
3.	F3	90.73 ± 0.12	78.26± 0.86
4	F4	92.00±0.61	76.12±0.48
5	F5	91.34±0.56	80.45±0.01

Each value represents the mean ± SD (n=3).

9.2.4 particle size analysis &Polydispersity index measurement

The particle size was measured by Malvern particle size analyzer. All the prepared formulations were in the nano size. The prepared nanoformulation shows nano size range from 87.68nm to 88.25nm and poly dispersity index range from 0.284 to 0.424. It indicates good uniformity in particle size distribution. The choice of suitable polymer and its concentration are most important factors to control the size and stability. Nanoformulation showed low particle size and poly dispersity index and formed a good formulation and it will indicate good stability of the nanoformulation.

This narrower range of particle size will minimize the difference between active agent concentration and the surrounding environment. As a result, the Ostwald ripening phenomenon will be inhibited in nanoformulation during mechanical shaking method.This

could be due to repeated circulation and prevention of RES uptake, which enables nanoparticle to be taken up by cancer cells.

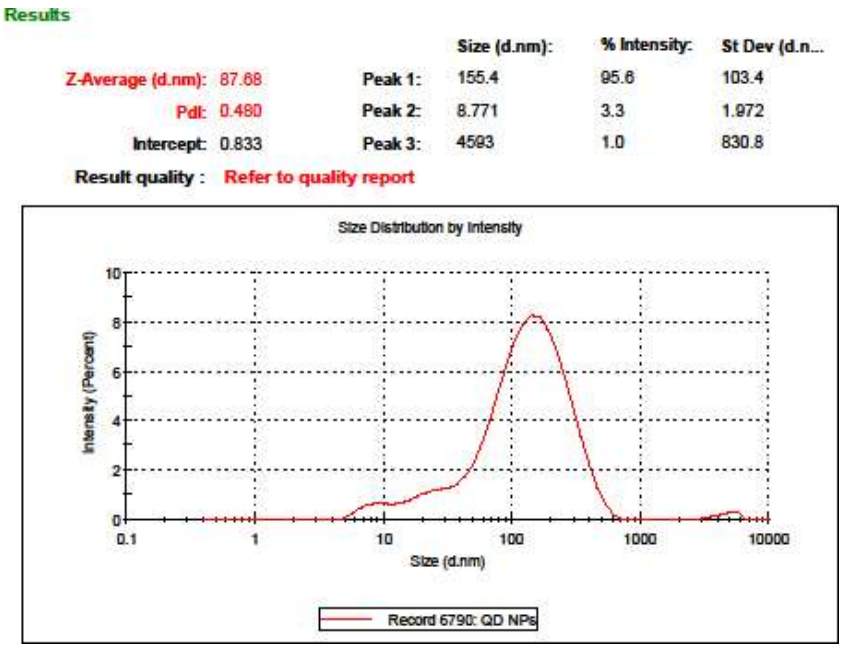

Fig.6 Particle size and Poly dispersity index of nanoformulation

Zeta potential analysis

The zeta potential had a foremost effect on the storage stability of colloid dispersion system and it reflected the electrostatic barriers which could intercept the nanoparticles from aggregation and agglomeration. As a rule of thumb, a zeta potential of at least −30 mV for electro statically or −20 mV for sterically stabilized systems is desired to obtain a physically stable nanoformulation. In the present study, the zeta potential of quercetin nanoformulation was found to possess negative values due to the negative charged soya lecithin and the measured zeta potential was low (-1.67 to -26.3 mV).

Soya lecithin was used to obtain the highest electric repulsion; thereby it increases the zeta potential between the particles and enhances the stability and saturation solubility. In addition, diffusion on the surface of drug nano particles was decreased and enhances the

concentration gradient thus leading to increase in the dissolution rate. So, it is adapted for both active and passive targeting in cancer therapy .Moreover, it was reported that negatively charged with hydrophilic polymer of nanoparticles results, escape the opsonization process irrespective of their particle size and an improved drug influx into cancer cell.

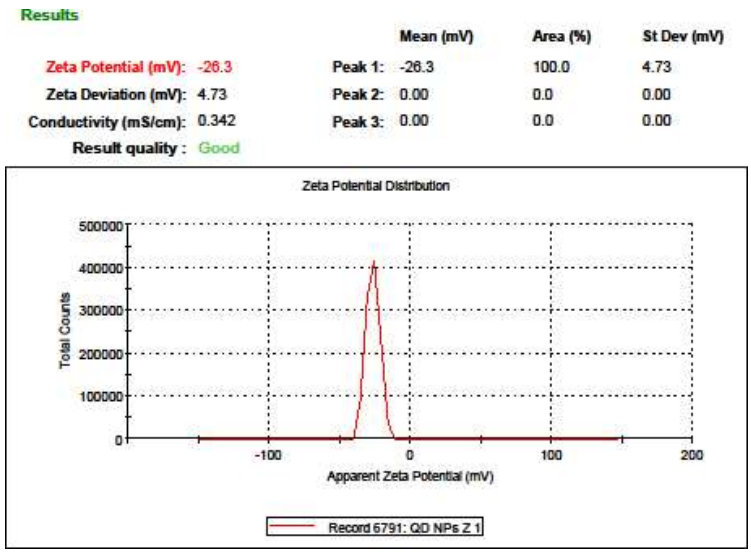

Fig.7 Zeta potential of Nanoformulation

Scanning electron microscopy

Scanning electron microscopy was carried out to study the surface morphology of particles. The SEM image of pure quercetin was found abundantly with larger particle size when compared to nanoformulation using chitosan, soya lecithin, cholesterol, produced better surface characteristics. The quercetin nanoformulation particles are smaller in size, among most of them are similar in dimensions. It was found that quercetin nanoformulation revealed a smooth texture with no polymeric aggregates. Hydrodynamic process takes place during the particle preparation which leads to the higher loading efficiency of quercetin in surrounding aqueous phase.

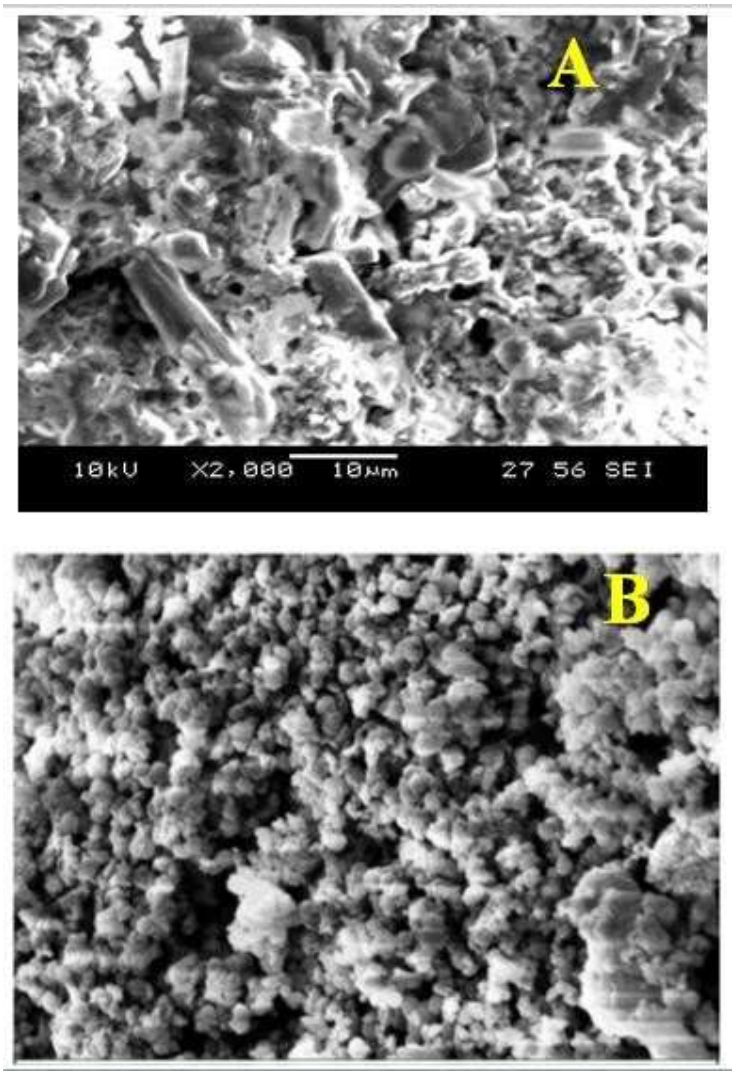

Fig.8 SEM image of quercetin dehydrate (pure drug) (A), SEM of quercetin loaded liposome nanoformulation (B).

Powder X-ray diffraction analysis (PXRD)

A powder X-ray diffraction method is useful tool in identifying the physical nature of the particles. Powder X-ray diffraction patterns of free quercetin, polymer and quercetin nanoformulation are present in Figure. Free quercetin has displayed the characteristic crystalline peaks of 2θ of 10.83°, 11.49°, 15.75°, 17.27°, 18.07°, 20.35°, 23.73°, 25.37° and 27.71° · However, quercetin nanoformulation has not showed any such crystalline peaks. The absence of detectable crystalline domains of quercetin in nanoformulation clearly indicates

that quercetin nanoformulation is in amorphous or disordered crystalline phase or in the solid solution state.

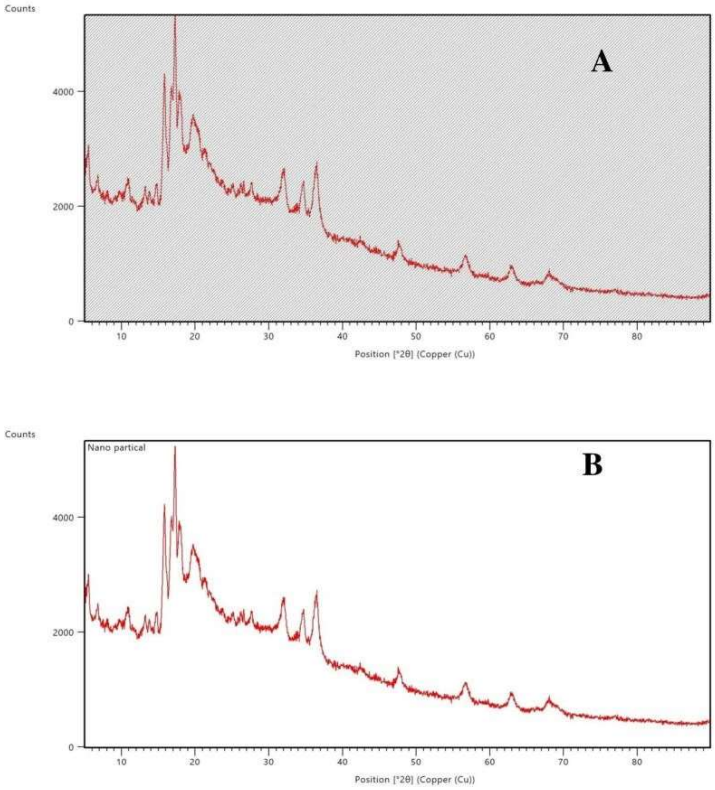

Fig.9 P-XRD of A. Quercetin pure drug and B. Quercetin loaded liposome nanoformulation.

Thermal analysis

Crystalline state is another criteria influencing dissolution and stability behavior of compounds. In general, an increase in saturation solubility and dissolution rate could be achieved by changing the crystalline state of the drug to amorphous . The mechanical shaking technology may induce changes in the crystalline state, such as increase of amorphous fraction or creation of completely amorphous particles. To investigate the molecular state of the quercetin, DSC study was carried out. The DSC curve of quercetin and nanoformulation were recorded. Figure Shows DSC thermograms of free quercetin, and quercetin nanoformulation. Free quercetin exhibited

a melting endothermic peak around at 315 to 316 ◦C indicating the crystalline nature of the drug.The result found for quercetin endorses those found in the literature. It could be seen that the melting point found in the analysis of DSC is similar to the melting range obtained by the capillary method for quercetin.

However, quercetin nanoformulation showed broad melting peak indicating the absence of crystalline. Nature of thermogram is totally changed and the sharp peaks are shifted, the peaks of pure quercetin have change to broad peaks with reduction of the height of each peak. These changes indicate that the dehydration of pure quercetin and change in the particle size giving more amorphous type of the product. It may helps in increasing the dissolution rate of nano formulation. Hence the nanoformulations were molecularly dispersed in an amorphous form.

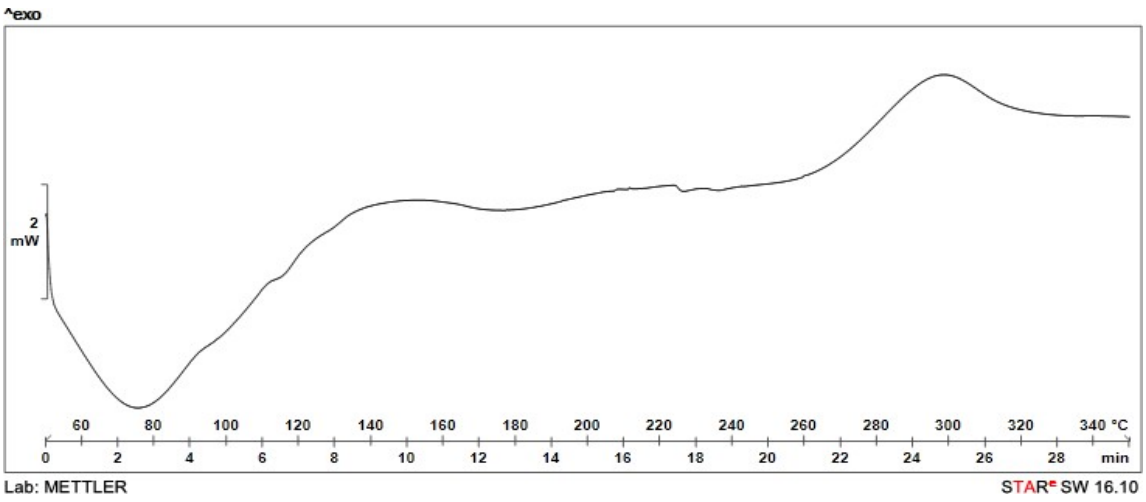

Fig. 10 DSC of Quercetin loaded liposome Nano formulation.

***IN-VITRO* DRUG RELEASE:**

In *in-vitro* release study, The cumulative percentage of drug release from quercetin quantum dot formulation F1-F5 shows 91.24%, 80.24%, 70.53%, 62.12% and 49.83% respectively, The dissolution studies of formulation F1 have shown 91.24% of drug release at the end of 12 hours in 0.1 HCL. This indicated the *in-vitro* release pattern of the formulations prepared happened to be sustained in comparison to that of the other formulations. The release of the formulation F5 was prolonged than the other formulation.

Table.7: Comparative *in-vitro* dissolution profile of pure drug and their formulation.

S. NO	TIME(min)	PURE	F1	F2	F3	F4	F5
1	0	0	0	0	0	0	0
2	30	1.941	23.62	14.23	10.35	6.79	4.53
3	60	3.882	29.76	20.38	15.53	10.67	7.44
4	90	5.500	33.65	25.56	19.41	12.94	9.06
5	120	6.147	37.53	27.82	23.29	15.53	10.35
6	180	7.118	42.71	34.29	28.47	19.09	12.61
7	240	9.383	47.24	39.15	33.65	22.00	18.76
8	360	11.64	66.00	52.41	42.38	33.65	25.23
9	480	24.26	73.77	68.59	56.94	46.27	39.47
10	600	30.41	86.71	77.01	67.95	57.59	46.59
11	720	38.18	91.24	80.24	70.53	62.12	49.83

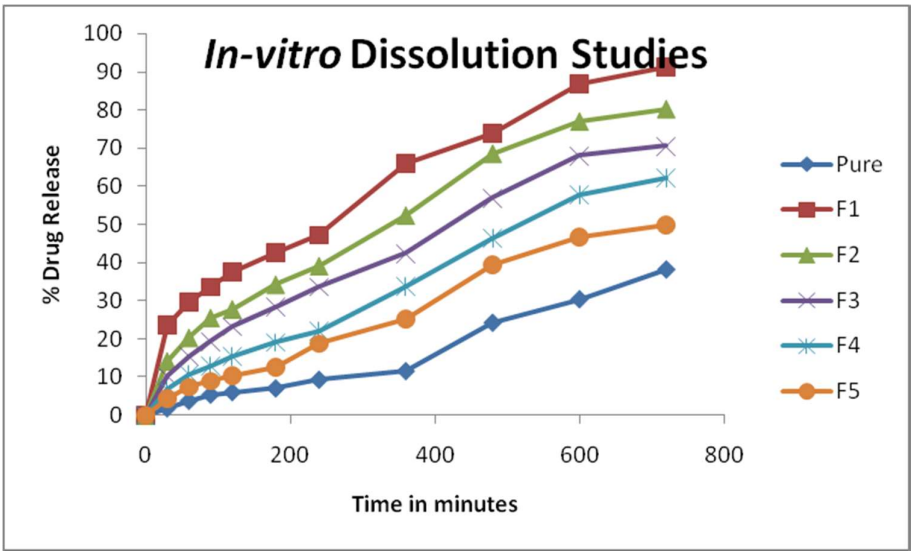

Fig 11: Comparative *in-vitro* dissolution profile of pure drug and their formulation.

***In-vitro* Drug release kinetic model profile:**

The *in-vitro* drug release data of the all sustained release tablet formulations were evaluated kinetically by zero order kinetics; first order kinetics, Higuchi plot and Peppas models. The F5 formulation shows maximum sustained drug release 49.83% at the end of 720 minutes. The regression coefficient value for Zero order, First order, Higuchi's, and Peppas plots for formulation F5 were found to be 0.9855, 0.9855, 0.8822, 0.9761 also plots were found to be linear, which indicates that the drug release depended on the square root of the time and predominantly controlled by diffusion process.

The mechanism of drug release is predicted by using Korsmeyer–Peppas equation. The n value of optimized formulation F5 was 0.78 and that of F5 formulation is less than 0.85. The optimized formulation F5 indicates that the drug release depends on swelling, diffusion, and erosion. All formulations follow the non-fickian or anomalous type of diffusion.

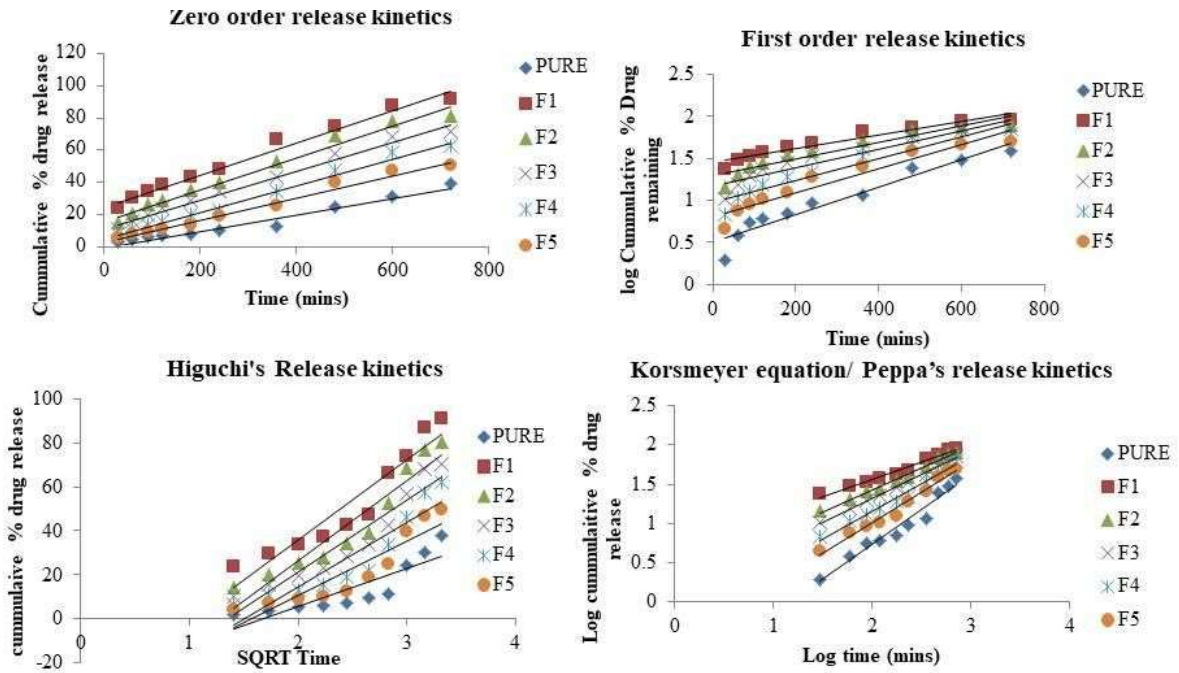

Fig 12 : Release kinetics of Pure drug and Formulations

Table.8 : *In-vitro* drug release kinetic profile of Quercetin F1-F5 formulations.

S.No	Formulation	Zero order reaction(r^2)	First order reaction(r^2)	Higuchi diffusion (r^2)	Korsemeyer/ peppas	
					(r^2)	n Values
1	F1	0.9846	0.9846	0.9318	0.9753	0.441
2	F2	0.9807	0.9807	0.9341	0.9897	0.558
3	F3	0.9855	0.9855	0.9312	0.9741	0.613
4	F4	0.9922	0.9922	0.8931	0.9805	0.706
5	F5	0.9855	0.9855	0.8822	0.9761	0.781

Invitro cell line study in glioblostoma cell line

Effects of Quercetin on viability of U87 cell line. Cells were treated with Quercetin (μM) for 72 hr. Controls were defined as cells treated with 0.1% DMSO without Quercetin. A: Effects of Quercetin on the proliferation of U87 cells were expressed as percentage of cell viability.

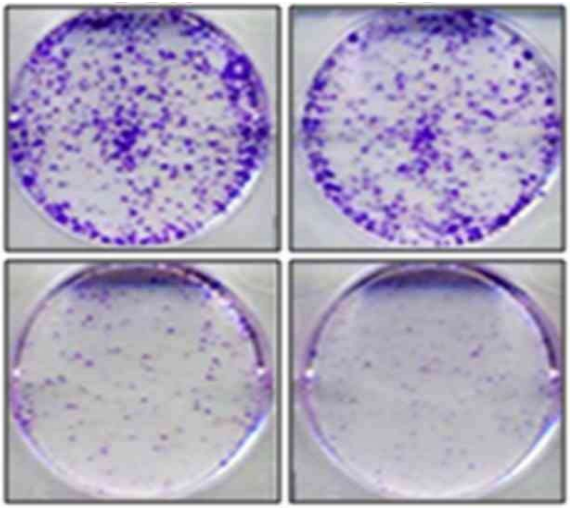

Fig.13: Image of cell degradation by adding the quercetin quantum dot loaded liposome nanoformulation

Stability studies

The promising quercetin formulation (F2) prepared from combination of soya lecithin and cholesterol were subjected to an accelerated stability studies using parameters like particle size, PDI, percentage of drug content and *in-vitro* release studies. The sample stored at 4 °C , exhibited optimum stability owing to the very low temperature.

At this temperature, the solubility of quercetin declined, leading to increased level of super saturation and rapid nucleation. Since the number of nuclei increased, the solute on each nuclei declined, and hence the potential for smaller crystals. Furthermore, low temperature decreases diffusion and growth kinetics at the particular boundary layer interface. It certified that the freeze dried powder possessed long-term stability which

attributed to no exist of Ostwald ripening occurring in the formulation and repulsions offered by the soya lecithin covering the surface of the particles.

The formulation at room temperature and at higher temperatures (40 °C) was showed slight increase in particle size. *In-vitro* dissolution profile data of the nanoformulation shows the dissolution rates of quercetin was satisfactory in 0.1N HCl and phosphate buffer. The percentage remaining drug content was magnificent after three month.

Table. 9 Particle size analysis of selected quercetin nanoformulation.

Formulation	Storage temperature condition	Initial particle size	Particle size after three months
F1	**4^0C**	80.52 ± 91.96	82.23 ± 24.34
	Room temperature		85.61 ± 95.40
	40^0C		92.02 ± 11.06

Each value represents the mean ± SD (n=3).

Conclusion:

Due to its weak solubility in aqueous solutions and low oral bioavailability, quercetin's clinical value is limited. Because conventional methods lacked cancer specificity, they failed in clinical development. With the help of a polymer like chitosan and a mechanical shaking technique, a novel dosage form called quercetin nanoformulation was successfully created to help with tumour localization and cancer-specific treatment. These innovative formulations have the potential to enhance quercetin's absorption, pass the blood-brain barrier, and offer a promising method of administration for the treatment of cancer. In comparison to quercetin, quercetin nanoformulation exhibits greater cytotoxicity in glioblastoma cells. This study shows a connection between quercetin's anti-proliferative effects and the induction of ROS-mediated mitochondrial membrane depolarization, nuclear condensation, and DNA fragmentation, which is a novel insight into the mechanism of action of quercetin induced in glioblostoma brain tumour cancer cells. The antioxidant and anti-cancer effects are boosted by the nanoformulation. As a result, the study's findings offer convincing support for the use of the quercetin-loaded liposome nanoformulation as a brain-targeting agent, chemotherapeutic agent, and cytotoxic agent against cancer cells in brain tumours. As a result, this novel formulation will significantly increase the effectiveness of future cancer therapies and enable more affordable cancer care. In future prospects the nanoformulation has to be delivered into the nasal to brain targeting method to target the brain tumor cells and it has to be target images and kill the tumor cell without affecting the normal cell by the active targeting method. To modify the surface of quantum dot and conjugate with the ligands for that future prospects study.

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