GSC Biological and Pharmaceutical Sciences
eISSN: 2581-3250 CODEN (USA): GBPSC2
Cross Ref DOI: 10.30574/gscbps
Journal homepage: https://gsconlinepress.com/journals/gscbps/

(RESEARCH ARTICLE)

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Nano-nutraceuticals: Formulation, evaluation, analytical method development and validation

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GSC Biological and Pharmaceutical Sciences, 2026, 35(03), 060-074

Publication history: Received on 25 April 2026; revised on 04 June 2026; accepted on 06 June 2026

Article DOI: https://doi.org/10.30574/gscbps.2026.35.3.0206

Abstract

In the United States, "nutraceutical" do not exist as a regulatory category; they are regulated as dietary supplements, food additives by the FDA, drug and cosmetic act.A lipid-based delivery system is one of feasible formulation techniques to encapsulate and improve the oral bioavailability of food bioactive compounds. the λ max of CoQ10, Curcumin and Resveratrol was found to be 274 nm, 430 nm and 307 nm respectively. The development of simultaneous estimation of the three nutraceuticals in the study requires determining the Isobestic point, and it was found to be 272 nm. The final fixed chromatographic conditions include, acetonitrile: 1%tetrahydrofuran: 1%acetic acid (70:20:10) at 1.0 ml/min flow rate as it shows highest resolution with minimum tailing effect. Retention time was 1.97 for CoQ 10, 3.16 min for Resveratrol and 4.89 min for Curcumin respectively. The results indicate all the nutraceutical formulation shows maximum drug release (76.8 to 80.9 %) and follows first order kinetics with the correlation coefficient in the range of 0.7 to 0.784. The criteria for a Nano formulation were established and it is evident from the SEM and AFM images.

Keywords: Λ Max; Chromatographic Conditions; Nano Formulation; SEM; AFM; Particle Size

1. Introduction

High pressure or high performance liquid chromatography is used in pharmaceutical industry for evaluations of a large variety of samples[1]. It is the method of choice for checking purity of new chemical entities, monitoring changes in synthetic procedures or scale up evaluating new formulations and carrying out quality control/assurance of the final product. Generally HPLC principle is based upon the adsorption techniques. Method validation is defined as process of demonstrating that analytical procedures are suitable for their intended use. Validation is the process by which it is established by laboratory studies that the performance characteristics of the method meet the requirements for the performance characteristics of the method meets the requirements for the intended application. Drugs which are poor water soluble are suitable candidate for lipid-based formulation.[2]

In the United States, "nutraceutical" do not exist as a regulatory category; they are regulated as dietary supplements, food additives by the FDA, drug and cosmetic act.

The reasons for shift towards nutraceuticals are increasing numbers of consumers, concerned about healthcare costs[3,4,5].

- Dissatisfied with pharmaceutical agents in promoting health, are turning to nutraceuticals to improve their health and prevent chronic disease.

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- Health care provider recognize the fact that our heavily processed food supply, coming from crops grown with chemical fertilizers, pesticides, herbicides, and often genetically modified seeds, lacks sufficient nutrients necessary for optimum health.[6,7]
- People believing more in prevention than a cure.
- People who have chronic diseases and have found no solution in allopathic medicines.

1.1. Selected Nutraceuticals

1.1.1. Resveratrol

Resveratrol is natural occurring phytochemical obtained from the roots of Polygonum cuspidatum and leaves of Veratrum grandiflorum belongs to the family Polyganaceae.

It belongs to class of phytochemical known as stilbenes and subclass phytoalexin [8].

1.1.2. Curcumin

Curcumin is a principal curcuminoid of the popular Indian spice turmeric, which is a member of ginger family (zingiberaceae).

It is a well-known fact that curcuminoids are natural phenols, a coloring principle in turmeric emerged as the most exploited Phyto-constituents for its wide spectrum of biological activities. [9]

1.1.3. Coenzyme Q10

Coenzyme Q10 is chemically 2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-benzoquinone, also known as ubiquinone which is fat soluble Quinone[10].

2. Materials and methods

The following materials were used in the study

Table 1 Materials Instruments

S. No	NAME	SOURCE
1	CoQ10, and Resveratrol	Sangrose Laboratories Pvt. Ltd. Kerala. India
2	Curcumin	Natural Remedies, Bangalore, India.
3	Potassium dihydrogen ortho phosphate	Himedia laboratories Pvt. Ltd
4	HPLC grade Methanol and Acetonitrile	Thermo Fischer Pvt. Ltd

Table 2 Instruments

S. No	Name of the instrument	Manufacturer
1	UV-Spectroscophotometer	Shimadzu (UV-1601 PC)
2	Differential Scanning Colorimetry	TA instruments (METTLER Toledo)
3	Atomic Force Microscope	Multimode Scanning probe microscope
4	Scanning Electron Microscope	JEOL Japan- JSM 6360
5	Digital pH meter	Systronis pH meter, LI-120, ELCO
6	High shear homogenizer	Pro Scientific- 250

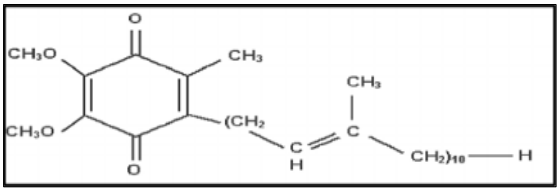
8	Particle size analyzer	Malvern Nano ZS-90
10	Solvent Degasser	Sonica Ultra sonic
12	High speed centrifuge	Eppendrof
13	Fluorescence Microscopy	Nikon Eclipse TS-100

2.1. Drug Profile

2.1.1. Coenzyme Q10

Coenzyme Q, also known as ubiquinone, is a coenzyme family that is ubiquitous in animals and most bacteria (hence the name ubiquinone). In humans, the most common form is coenzyme Q10 or ubiquinone-10[11].

Structure



Molecular weight : 863.3g/mol

Molecular formula : C59H90O4

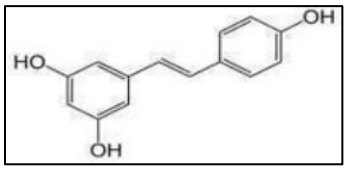
Physical form : Yellow to Orange solid

Solubility : soluble in acetone, chloroform and THF, sparingly soluble in acetonitrile and formic acid

2.1.2. Resveratrol

Resveratrol is a phytoalexin that belongs to the group of compounds known as stilbenes, and is known to occur in grapes, grape products and in wine. It is present in higher concentration in red grape varieties[12].

Structure



Molecular weight : 228.25 g/mol-1

Molecular formula : C14H12O3

Physical form : off white powder

Solubility : soluble in water, ethanol, methanol and acetone

2.1.3. Curcumin

Curcumin is a beta-diketone that is methane in which two of the hydrogen's are substituted by feruloyl groups. This is a natural dyestuff found in the root of Curcuma longa[13].

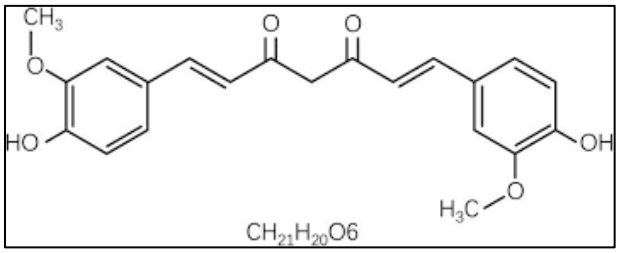
Product : Turmeric oleoresin 95% curcumin

Formula: C21H20O6

Molecular weight : 368.39 g/mol-1

Solubility : Insoluble in water and ether; soluble in organic solvent methanol, ethanol, chloroform, ether

Structure



3. Results and analysis

3.1. Solubility of Nutraceuticals[14,15,16]

3.1.1. Schefter and Higuchi Method

The Solubility of CoQ10, Curcumin and Resveratrol were determined in various solvents by using Schefter and Higuchi method.

Table 3 Solubility of Nutraceuticals in various solvents

Solvents	Coq10	Curcumin	Resveratrol
Methanol	Insoluble	Soluble	Soluble
THF	Well Soluble	Soluble	Well Soluble
Acetonitrile	Slightly Soluble	Slightly Soluble	Soluble
Acetone	Well Soluble	Soluble	Soluble

3.1.2. Assay Method UV- Spectrophotometer (UV-1601 PC Shimadzu, Japan)

Stock solution was prepared, and serial dilution was made. Different concentrations were chosen for determination of λ max which shows maximum absorption at 430 nm for curcumin, 308 nm for resveratrol and 274 nm for coenzyme Q10.

3.2. Determination of Isobestic Point: The Isobestic point was found to be 272 nm.

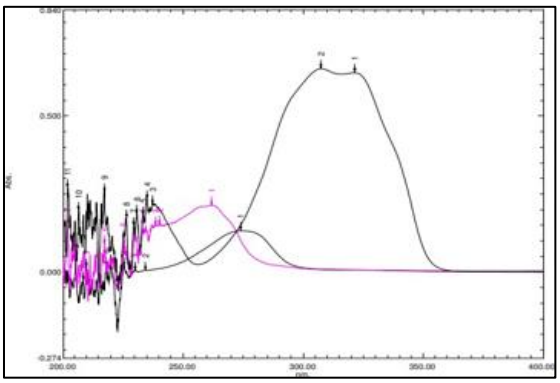


Figure 1 Overlay spectrum of Resveratrol, COQ 10

3.3. Fourier Transform Infrared Spectroscopy:

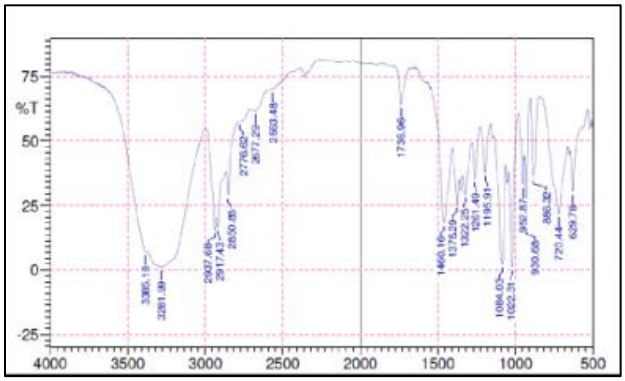


Figure 2 IR Spectrum of combined Formulation (Resveratol, Curcumin and CoQ10)

3.4. Differential Scanning Calorimeter

The DSC Curves shows that the melting point of the nutraceuticals is reduced when compared to the pure nutraceuticals and there is no interaction between the nutraceutical and excipients.

3.5. Analytical Method Development and Validation of Resveratrol, Curcumin and CoQ10[17,18]

Optimized chromatographic conditions.

Mode of separation : Isocratic

Mobile phase : Acetonitrile: 1% Tetrahydrofuran: 1% Acetic acid (70:20:10) Column: Sun fire (150×4.6mm, 5µm)

Detection wavelength: 272 nm

Run time : 10 min

Injection volume: 20µl

Flow rate : 1 ml/min

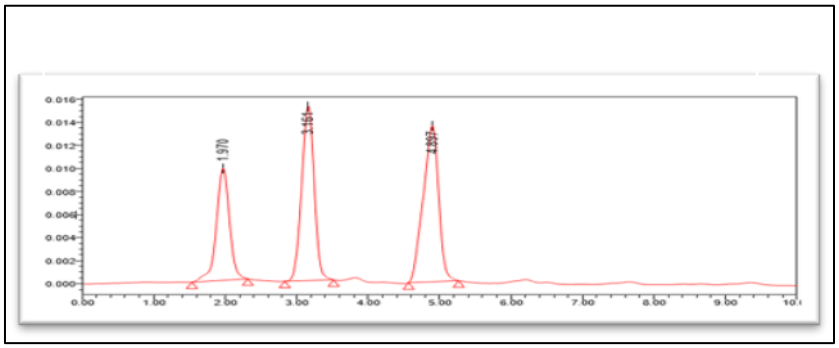


Figure 3 Standard chromatogram of Resveratrol, Curcumin and CoQ1

3.6. Method Validation of Resveratrol, Curcumin and CoQ10

3.6.1. Linearity and Range

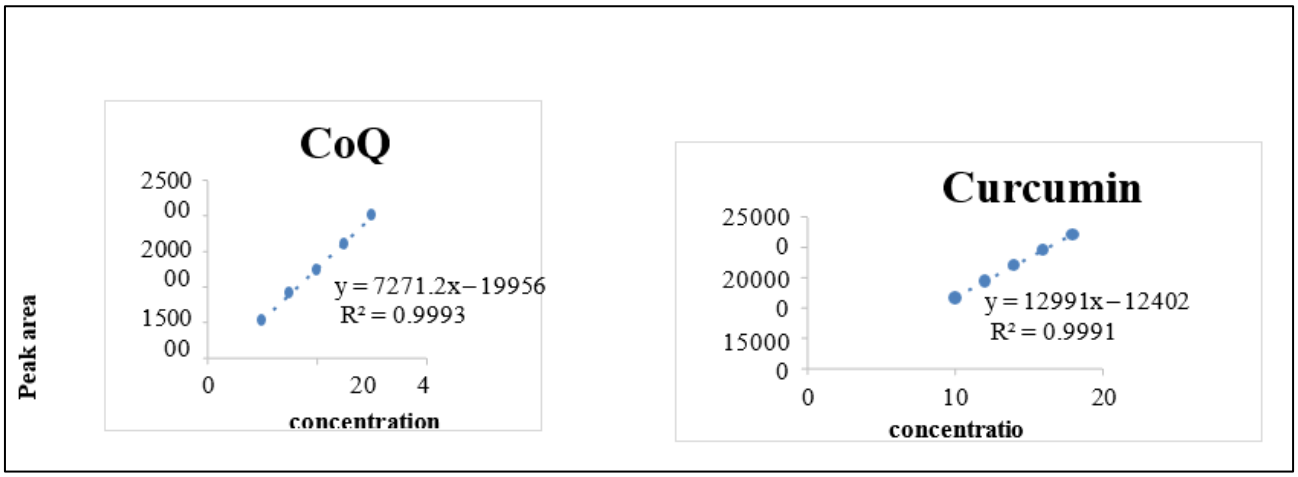


Figure 4 Linearity graph of CoQ10 and Linearity graph of Curcumin

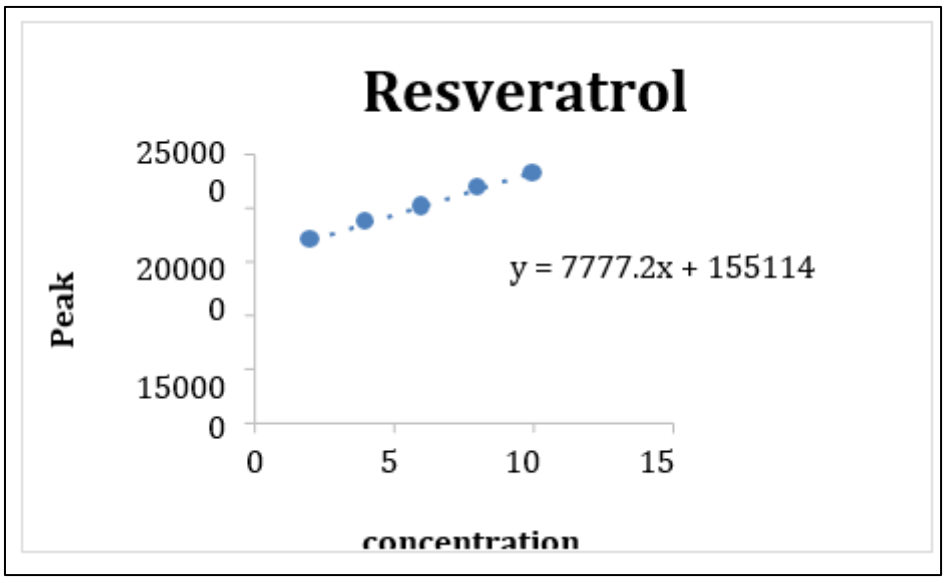


Figure 5 Linearity graph of Resveratrol

Table 4 Intraday precision

Level	Concentration (µg/ml)			Peak area			%RSD		
	CoQ10	Curcumin	Resveratrol	CoQ10	Curcumin	Resveratrol	CoQ10	Curcumin	Resveratrol
1	15	12	4	91412	143952	187459	0.3	0.2	0.3
				90767	143447	186936			
				90831	144025	186241			
2	20	14	6	124001	160336	202813	0.4	0.5	0.5
				123787	161941	203187			
				124852	160818	205046			

3	25	16	8	161138	196552	218567	0.7	0.2	0.3
				163587	196138	217694			
				162194	195597	218995			

Table 5 Intraday Precision of CoQ 10 , Curcumin & Resveratrol Interday precision

Level	**Concentration (µg/ml)**			**Peak area**			**%RSD**		
	CoQ10	Curcumin	Resveratrol	CoQ10	Curcumin	Resveratrol	CoQ10	Curcumin	Resveratrol
1	15	12	4	90536	143654	186589	0.2	0.5	0.2
				91064	145251	185947			
				90783	144762	186894			
2	20	14	6	126085	182612	203789	0.4	0.7	0.6
				125301	179965	206231			
				124986	180863	205497			
3	25	16	8	161125	196074	219532	0.2	0.3	0.3
				160384	195789	218567			
				160627	197126	219841			

Table 6 Interday Precision of CoQ10, Curcumin & Resveratrol Repeatability

Concentration (µg/ml)			**Peak area**			**%RSD**		
CoQ10	Curcumin	Resveratrol	CoQ10	Curcumin	Resveratrol	CoQ10	Curcumin	Resveratrol
20	14	6	123975	180365	202994	0.4	0.7	0.3
			124031	178742	204621			
			124453	181097	203127			
			125164	179489	203965			
			124252	178285	203548			
			125306	177634	204064			

Table 7 Repeatability of CoQ10, Curcumin & Resveratrol Accuracy

S. No	**Level**	**%Recovery**			**%RSD**		
		CoQ10	Curcumin	Resveratrol	CoQ10	Curcumin	Resveratrol
1	80%	101.1	99.97	99.9	100.17	99.77	99.74
2	100%	100.02	99.56	99.35			
3	120%	99.4	99.78	99.97			

Table 8 Recovery Studies of CoQ10, Curcumin & Resveratrol

Limit of Detection (LOD) & Limit of Quantification (LOQ)		
Drugs	Parameters	
	LOD	LOQ
CoQ10	0.811	2.459
Curcumin	0.364	1.104
Resveratrol	0.187	0.569

3.7. System Suitability[19]

System suitability was not more than 2% for CoQ10, Curcumin and Resveratrol. The results obtained are within the acceptance criteria.

Table 9 System Suitability parameters of CoQ10, Curcumin & Resveratrol

Parameters	**CoQ10**	**Curcumin**	**Resveratrol**
Linearity range	10-30 µg/ml	10-18 µg/ml	2-10 µg/ml
Retention time	1.97 min	3.16 min	4.89 min
Number of theoretical plates	8231	11436	9452
Correlation co-efficient	0.9993	0.9991	0.999
LOD	0.811	0.364	0.187
LOQ	2.459	1.104	0.569

3.8. Preparation of Solid Lipid Nanoparticles

Solid lipid Nano particles were prepared by hot homogenization method using high shear homogenization and probe sonication.

3.9. Standard Curve for Resveratrol, Coenzyme Q10 and Curcumin by Phosphate Buffer

Phosphate buffer pH 7.4 was used as the buffering medium in the preparation of standard solution of Resveratrol curcumin and coenzyme Q10.

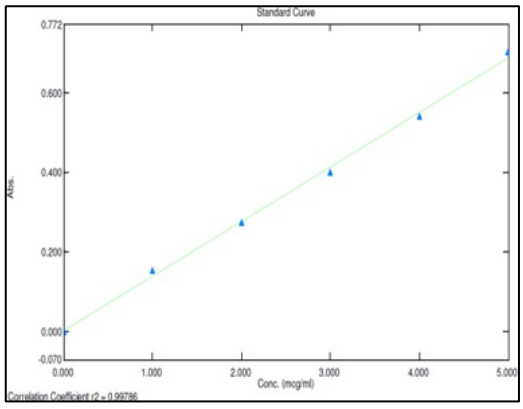


Figure 6 Standard graph for Resveratrol

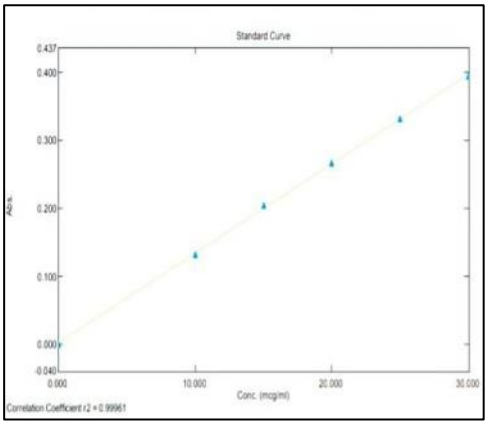


Figure 7 Standard graph for Coenzyme Q10

3.10. Resveratrol, CoQ10 and Curcumin

The following Nano formulations were selected for Resveratrol, CoQ10 and Curcumin and the best formulations were evaluated in each trial

Table 10 Evaluation Resveratrol & Curcumin (R+CUR F)

S. No	Formulation code	Particle size	Zeta potential (mV)	PDI
1	R+C+CUR F1	227.5	-5.5.3	0.342
2	R+C+CUR F2	190.7	-32.3	0.280
3	R+C+CUR F3	252.5	-12.3	0.820

From the above table, the formulation with the code R+C+CUR F2 was selected as the best formulation among the selected batch as it shows less particle size of 190.7 nm, with the Zeta potential -32.3 mV and PDI of 0.280 compared to the other two formulations.

3.11. Identification of the Physical Form the Drug in the Nanoparticles by DSC Analysis:

DSC study is performed to characterize the physical state of the entrapped drug in nanoparticles and to find out the nature of the lipid. Any instabilities and interactions among the drug and excipients can also be found out.

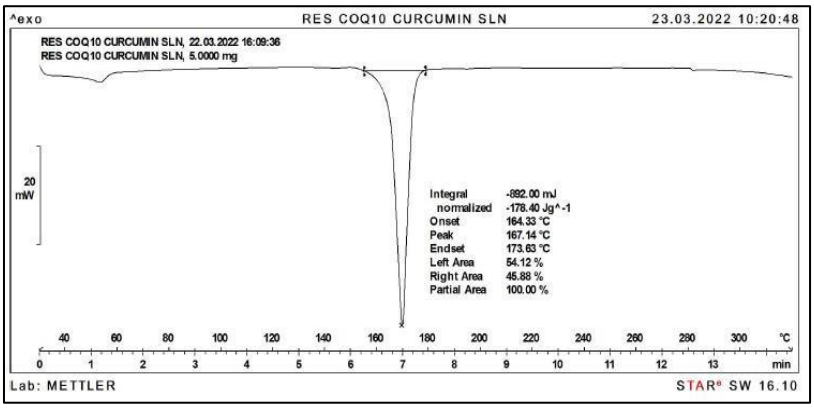


Figure 8 R+C+CUR F2 DSC Curve

The DSC Curves suggests that the melting point of the nutraceuticals is reduced while compared to the pure nutraceuticals. Therefore the nutraceuticals are converted as amorphous form with reduced crystallinity which may increase the solubility of the nutraceuticals by SLN formulations

3.12. Measurement of Particle Size and Surface Charge by Malvern Particle Size Analyzer

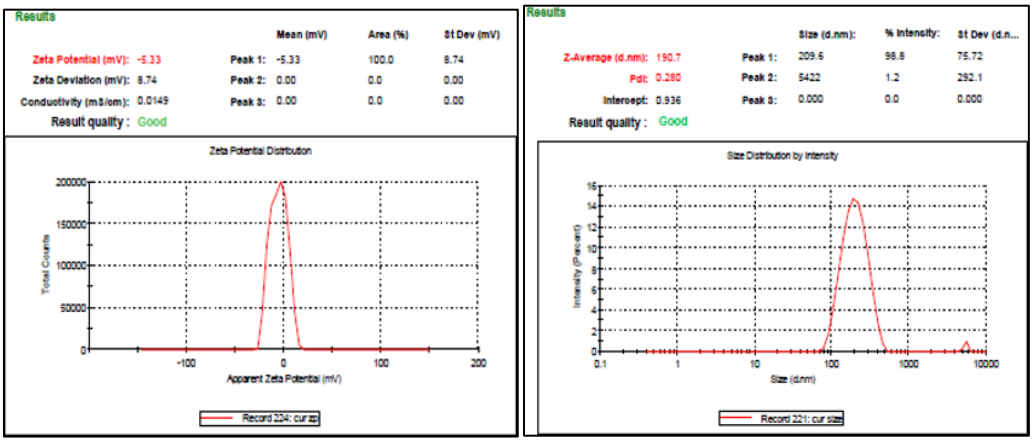


Figure 9 RES+CUR+CoQ10 Size and Zeta Potential

3.13. Entrapment Efficiency

The entrapment efficiency of prepared solid lipid nanoparticles was determined by ultra-centrifugation technique. 2 ml of the formulation was centrifuged at a speed of 13,000 rpm (107,662*g) for 1hr at 4°C.

Table 11 Entrapment efficiency of the developed SLN Formulations loaded with Nutraceuticals

S. No	Formulation Code	%EE						
		RF2	CF1	CUR F3	C + CUR F3	R + CUR F2	R + C F3	R + C + CUR F 2
1	Resveratrol	79.21				70.32	73.65	69.23
2	Curcumin			71.32	68.32	75.21		72.12
3	CoQ10		74.96		75.81		71.59	74.98

3.14. In-Vitro Drug Release Studies:[20]

The *in vitro* drug release studies will carry out by using dialysis bag method. 2 ml of drug formulation is transferred into the dialysis bag and both the ends are sealed tightly. That sealed dialysis bag will be incubated in a beaker containing phosphate buffer solution of pH 7.4 at 37 ± 2º C and stirred at 50 rpm.

Table 12 *In vitro* release studies

Tim (Mins)	% Drug Release						
	C F1	CUR F3	R F2	R+C F3	C+CUR F3	R+CUR F2	R+C+CUR F2
0	0	0	0	0	0	0	0
5	21.421	21.190	21.306	21.306	22.921	21.190	21.433
10	24.041	22.883	23.347	22.770	23.610	23.229	22.900
15	39.279	38.44	40.647	36.137	37.339	39.37	37.526
30	41.316	42.887	42.481	41.803	40.836	42.220	41.951
60	44.877	46.019	46.065	44.799	44.850	46.492	44.603
120	49.302	52.774	53.166	49.800	52.735	52.795	50.984
180	60.950	66.79	67.313	58.576	64.452	65.896	55.168
240	69.236	73.931	75.493	62.892	71.539	73.818	68.184
480	73.513	78.413	78.505	73.275	74.821	77.721	74.981
720	78.196	82.035	82.127	76.804	79.527	80.867	80.965

The formulations follow first order kinetics with the correlation coefficient in the range of 0.7 to 0.784. Release mechanisms of formulations exhibit higuchi model with the values at the maximum of 0.939, so mechanism of release is correlated with the koresmeyer peppas model. The n value in the peppas model was found to be less than 0.5 for the formulations which confirm the quantification diffusion. Thus all the formulations follow diffusion of drug with non swellable type of SLN drug delivery.

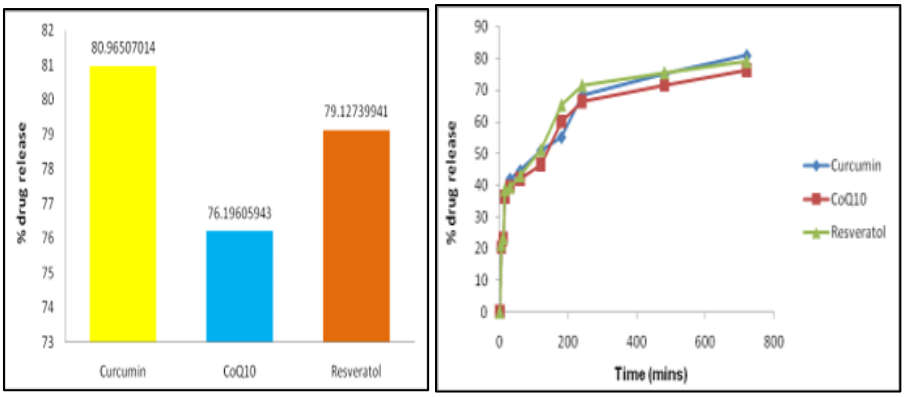


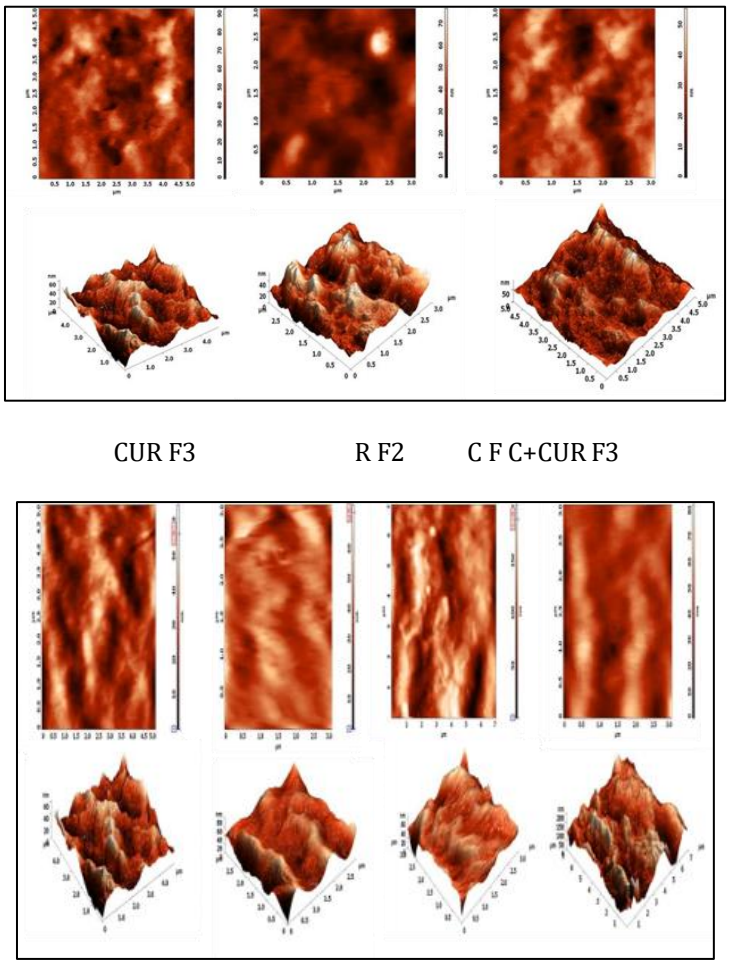
Figure 10 R+C+CUR F2 SLN release profile

3.15. Identification of Particle Shape and Morphology by SEM and AFM

3.15.1. Atomic Force Microscope (AFM)

10µl of samples were deposited on the surface of a silicon wafer and allowed to dry at room temperature to identify under AFM. In semi-contact mode, the morphology of the SLN was characterized using a Multimode scanning probe microscope (NTMDT, NTEGRA Prima and Russia).

3.15.2. AFM Results for Study of Morphology



CUR F3 R F2 C F C+CUR F3

R+C F3 R+CUR F2 R+C+CUR F2

Figure 11 AFM Results for study of morphology in nutraceutical formulation

3.16. Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM; Zeiss Sigma, Germany) was performed to analyze the morphology of the Nano formulations

R+CUR F2 R+C+CUR F2

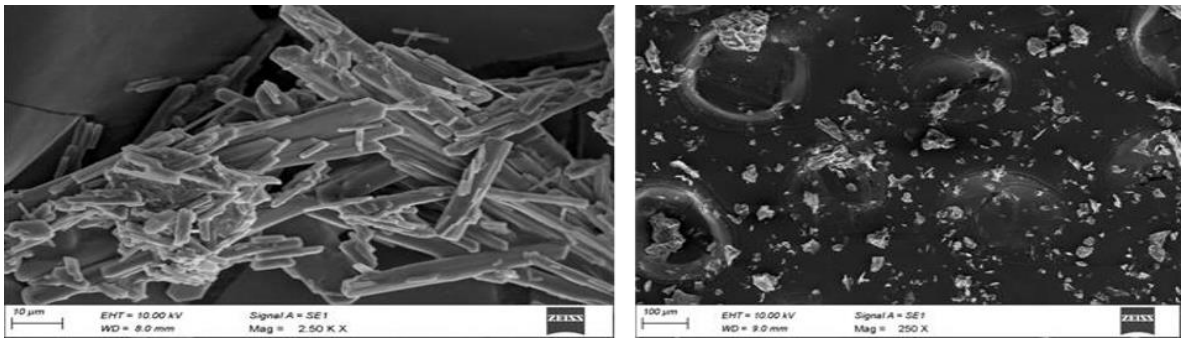


Figure 12 SEM Images for Combination of Nutraceuticals

SEM and AFM micrographs of Nano formulations indicate that the formed particles are spherical and mono dispersed with a particle size in range of nanometer. No change in the morphology. The particle size was in agreement with the values obtained by DLS. The images of Nano formulations clearly showed that the formulations were free of aggregations.

3.17. Stability studies[21,22]

Stability studies for Nano therapeutics will be evaluated as per ICH guidelines for 90 days in stability chamber. Periodically the particle size, zeta potential and PDI will be evaluated.

Table 13 Stability data of the best formulations (Room Temperature)

Storage Conditions	Formulation Code	Duration (Month)	Size (d.nm)	PDI	Zeta Potential (mV)
Time of Preparation	R+C+CUR F2	0	190.7	0.280	-32.3
Room Temperature (23°±0.2C)	R+C+CUR F2	3	189.1	0.278	-31.9
		6	196.6	0.281	-30.6
		12	204.9	0.289	-30

Table 14 Stability data of the best formulations (Refrigeration Temperature)

Storage Conditions	Formulation Code	Duration (Month)	Size (d. nm)	PDI	Zeta Potential (mV)
Refrigerator Temperature (4°±0.1C)	R+C+CUR F2	3	182.6	0.267	-27.0
		6	185.4	0.273	-28.2
		12	189.1	0.278	-30.5

4. Discussion

4.1. Preformulation Studies.

4.1.1. Solubility

Curcumin, Resveratrol and Coenzyme Q10 were found to be well soluble in tetra hydro furan and acetonitrile.

4.1.2. The UV spectroscopic Analysis

The λ max of CoQ10, Curcumin and Resveratrol was found to be 274 nm, 430 nm and 307 nm respectively. The development of simultaneous estimation of the three nutraceuticals in the study requires determining the Isobestic point, and it was found to be 272 nm.

4.1.3. The DSC analysis

The DSC analysis showed that there is no interaction between the drugs and excipients which is evident from the DSC curves comparison between the standard nutraceuticals. Hence the Preformulation data signifies the purity and compatibility of the raw materials involved in preparation of Nano formulations.

4.1.4. Analytical Method Development and Validation of Resveratrol, Curcumin and CoQ 10

The final fixed chromatographic conditions include, acetonitrile: 1%tetrahydrofuran: 1%acetic acid (70:20:10) at 1.0 ml/min flow rate as it shows highest resolution with minimum tailing effect. Retention time was 1.97 for CoQ 10, 3.16 min for Resveratrol and 4.89 min for Curcumin respectively.

4.2. Preparation of solid lipid nanoparticles and nutraceuticals

Solid lipid nanoparticles of the various characteristic nutraceutical formulation such as Resveratrol (R F), CoQ10 (C F), Curcumin (Cur F), CoQ10+Curcumin (C+Cur F), Resveratrol+Coq10 (R+C F) Resveratrol+Curcumin (R+Cur F), Resveratrol+Coq10+Curcumin (R+C+Cur F) was developed by hot homogenization method using high shear homogenization followed by probe sonication. Based on the trial studies, various proportional ratios of nutraceuticals, lipid (Glyceryl monostearate-GMS), surfactant (Brij72%) and co-surfactant (Brij 35%) was fixed. The best formulations were selected based on particle size, zeta potential and PDI.

4.3. Characterization

4.3.1. Particle size, Zeta potential, PDI

The prepared formulations were characterized and the particle size, zeta potential, PDI of (Resveratrol [R F2], CoQ10 [C F1] Curcumin [Cur F3], oQ10+Curcumin[C+CurF3],Resveratrol+Coq10[R+CF3],Resveratrol+Curcumin[R+CurF2],Resveratrol+Coq10+Curcumin [R+C+Cur F2]) were found to be having ideal properties in the range of particle size (132.9 to 325.21 nm) zeta potential (-22.6 to -36.9 Mv) PDI (0.212 to 0.450). DSC

4.4. Entrapment Efficiency

The observed entrapment efficiency of (Resveratrol [R F2], CoQ10 [C F1] Curcumin [Cur F3], CoQ10+Curcumin [C+Cur F3], Resveratrol+Coq10 [R+C F3], Resveratrol+Curcumin [R+Cur F2], Resveratrol+Coq10+Curcumin [R+C+Cur F2]) was found to be in the range (68.32 to 79.21% W/W). The obtained results indicate, the nutraceuticals incorporated in the nanoparticles matrix system (SLNs) shows optimum distribution of drug particles.

4.5. *In-vitro* drug release studies

The results indicate all the nutraceutical formulation shows maximum drug release (76.8 to 80.9 %) and follows first order kinetics with the correlation coefficient in the range of 0.7 to 0.784.

4.6. Identification of particle shape and morphology by SEM and AFM

The spherical shape particles are evident from the images and the particles are free of aggregation. The criteria for a nano formulation were established and it is evident from the SEM and AFM images.

4.7. Stability studies

On the other hand formulations (Resveratrol [R F2], CoQ10 [C F1] Curcumin [Cur F3], CoQ10+Curcumin [C+Cur F3], Resveratrol+Coq10 [R+C F3], Resveratrol+Curcumin [R+Cur F2], Resveratrol+Coq10+Curcumin [R+C+Cur F2]) stored at refrigerator temperature (4^{o}C) shown stable particle size, PDI and zeta potential at 3rd, 6th and 12th month and was found to have stability under the storage conditions.

Compliance with ethical standards

Acknowledgments

The authors wish to thank Director KYDSCT COP, Sakegaon Bhusawal for their willing support and constant encouragement.

Disclosure of conflict of interest

The authors declare no conflicts of interest, financial or otherwise. Availability of data and materials Supplementary material that is available on the publisher's website along with the published article contains spectroscopic data of all compounds.

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