AMERICAN JOURNAL OF PHARMTECH RESEARCH

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Development of Naringenin Nanocrystals for Enhanced Solubility and Bioavailability

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ABSTRACT

Naringenin is a flavonoid which has been used for its wide pharmacological action from ancient years including as antidiabetic agent. Naringenin is a lipophilic drug (BCS-II) and have low water solubility (1 in 1000), bioavailability (<25%) and have short half-life ($t_{1/2}$ =1.3 -2.2h). Nanocrystals is an approach to increase the therapeutic performance of poorly water soluble drugs. The purpose of the present study was to prepare nanocrystalss of naringenin to improve bioavailability and increase therapeutic efficacy. Nanocrystalss of naringenin were prepared by antisolvent precipitation method. The stabilizers used to improve aggregation and increase the solubility. Nanocrystals were characterized for particle size, morphology, release profile and thermal analysis.

Keywords: Naringenin, Nanocrystals, Bioavailability, Solubility, antisolvent precipitation.

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Received 01 February 2018, Accepted 23 February 2018

Please cite this article as: Singh MN *et al.*, Development of Naringenin Nanocrystals for Enhanced Solubility and Bioavailability. American Journal of PharmTech Research 2018.

INTRODUCTION

Naringenin is BCS II drug with poor aqueous solubility and low bioavailability (<25%). The drug is also facing short half-life (1.3-2.2 h). Naringenin is a flavonone that is considered to have a bioactive effect on human health as antioxidant, radical scavenger, antiinflammatory, carbohydrate metabolism promoter, immunity system modulater. Recent investigation suggested that nanocrystals form of drug has good water solubility and therefore have higher bioavilability[1].

Formulation of nanocrystals will overcome the above stated problems. The nanocrystalss are supposed to enhance the aqueous solubility as well as dissolution rate of the drug, which may lead to increased bioavailability.

Nowadays nanotechnology offers various approaches in the area of dissolution enhancement of poor water soluble drugs. Nanosizing of drugs in the form of nanoparticles, Nano crystals or nanosuspensions to increase the bioavailability and dissolution rate of poorly water soluble drugs.[1,2]

Poorly water soluble drugs are often a various challenging problem in drug formulation. Reducing the particle size of the drug to a nanosize leads to an increased surface area-to-volume ratio, increased dissolution velocity and adhesiveness, and improved *in vivo* performance of poorly water soluble drugs[3]. The poor solubility of drug is a major problem which limits the development of highly potent pharmaceutics. The drugs with low solubility lead to low oral bioavailability and erratic absorption which is particularly pertinent to drugs within class II of the Biopharmaceutical Classification System (BCS). Nanocrystalss of these drugs are generally prepared to improve the drug solubility in order to enhance the bioavailability. Drug nanocrystalss are pure solid drug particles with a mean diameter below 1000 nm.[4,5]

The techniques to produce drug nanocrystalss can be divided in two basic approaches, namely the bottom up and the top down technologies. The top down techniques are based on size reduction of relatively large particles into smaller particles by mechanical attrition whereas bottom up techniques consists of growth of smaller particle from individual molecules. The driving force for the growth of a crystal from individual molecules is supersaturation. Super saturation of a drug in a solution can be obtained by decreasing the temperature or addition of an anti-solvent.[6,7,8]

MATERIALS AND METHOD

Materials

Naringenin was purchased from MP LLC. Mannitol was purchased from Himedia Laboratories

Ltd. (Mumbai, India). All other reagents and solvents used were of analytical grade. In house distilled water was used throughout the experiment.

Animal

The *in vivo* study was performed according to the protocol approved by the Institutional Animal Ethics Committee as per approval (reference number AEC/PHARM/1601/06/2016/R2). For the study, 72 albino wistar rats weighing 80-120 g were acclimatized under maintained laboratory conditions at ambient temperature of 25±2°C and relative humidity of 50±5% with a 12 h light/12 h dark cycle.

Antisolvent Precipitation Method[9,10,11]

Nanocrystals of Naringenin drug was prepared by Bottom up precipitation method. In this method no stabilizer is used. These were prepared by controlled crystallization during freeze drying. In this method mannitol is used as a carrier. It easily crystallizes during freeze drying. Two separate solutions of drug in Dimethyl sulfoxide and mannitol in water were prepared and heated upto 60ºC. Aqueous solution was mixed in drug solution. Instantly after mixing, the solution was frozen and thereafter dried in vacuum desiccators. Before employing this method some trial batches were prepared by this method using different drying system like lyophilizer, rotary evaporator and vacuum desiccators. The crystals obtained from vacuum desicator were shown better morphology as compared to other drying method. So desicator drying method was employed.

Table 1: Formulation of nanocrystals formulation

S.no.	Formulation code	Mannitol (mg)	Drug (mg)	DMSO (mL)	Water (mL)
1.	F1	1800	80	4	6
2.	F2	1200	80	4	6
3.	F3	600	80	4	6
4.	F4	300	80	4	6

Characterisation of Naringenin nanocrystals

Drug excipient compatibility[12 ,13]

IR spectra of pure cholchicine and formulation (drug loaded chitosan nanoparticle) were recorded using FTIR (Nicolet^{TM} 6700, Thermo Scientific USA) spectrophotometer in a frequency range 4000-350cm^{-1} with 100 number scan and 4 cm^{-1} spectral resolution (Mehta, 2013). IR spectra were recorded on Chitosan nanoparticles loaded with colchicine or nanoparticles alone mixed with KBR (1%w/w nanoparticles) and pressed to plate for measurement.

Particle size, polydispersity index (PDI) and zeta potential

The NAR nanocrystalss were characterized for morphology viz. particle size, zeta potential and PDI. The average particle size and PDI of nanoparticles were evaluated by photon correlation spectroscopy zeta sizer nanoplus-3 (Japan). All the samples were suitably diluted and analysed using 1ml cuvette in a thermostatic chamber at 25^0C using a He-Ne laser. All the experiments were done in triplicate [14].

Drug content [15,16,17]

Drug content was determined by dissolving Naringenin nanocrystalss (NARNC) formulation in methanol and stirred for 15 min. Samples were filtered through syringe filter. Drug content was determined spectrophotometrically (UV-Vis spectrophotometer Labtronics LT2910) at 285nm after suitable dilution. The samples were diluted using methanol prior to absorbance analysis. All the experiments were performed in triplicate.

Entrapment efficiency [16]

The formulation mixture were centrifuged at 10,000 rpm for 15 min. The supernatant solution was collected and the absorbance was measured using UV spectrophotometer (UV-Vis spectrophotometer Labtronics LT2910) at 285nm. The amount of unentrapped drug in the supernatant was calculated, using formula

Entrapment Efficiency (%) = Total amt. of drug – free unentrapped drug/ Total amt. of drug X 100

Particle Morphology SEM (Scanning Electron Microscope) [18]

The morphology and surface characterization of nanocrystalss were examined by using scanning electron microscope model JSM-6490LV (JEOL, Japan). Before examinations the samples were mounted on top of double sided sticky carbon tape on metal discs and coated with 80nm gold/palladium in Balzers 120B sputtering device.

Differential Scanning calorimetry (DSC) [19]

DSC analysis was carried out using differential scanning calorimetry model Pyris Diamond TG/DTA PerkinElmer (Singapore). Samples (pure drug, mannitol and nanocrystalss formulation) of about 3 mg were weighed accurately and put in aluminum pan and sealed in a lid. Heat runs for each sample has been set from 50 to 300° at a Scanning rate of 10° min-1, under dry nitrogen flow (150 ml/min). The apparatus is indium/cyclohexane calibrated.

X-ray powder diffraction (XRD)

Samples (Naringenin drug, excipient and nanocrystalss formulation) were evaluated with X-ray diffractometer: Model- Ultima-III, Rigaku make (Japan), Cu target slit 10 mm. Standard runs using

a 40 kV voltage, a 2mA current. The scanning rate employed was 10 min-1 over the 10 to 300 diffraction angle (2θ) range.[20,21,22]

Solubility Analysis

The solubility was determined by a shake-flask method. Solubility of naringenin nanocrystals formulation were tested in different solvents such as distilled water, buffer (pH 1.2, 4.0, 6.8, and 7.4). An excess amount of naringenin nanocrystals was added in 2ml of the pertinent solvents. The mixtures were stirred in a mechanical shaker for 72 hours. Visual inspection was carefully made to ensure there were excess solids in the mixture, indicating saturation had been reached. The mixtures were then centrifuged, filtered and filtrates were diluted suitably to determine the solubility of naringenin nanocrystals in each solvent under UV spectrophotometer at 285 nm.[23,24]

In-Vitro Dissolution Study [25,26]

The release rate of naringenin nanocrystals was determined using USP Dissolution testing apparatus II (Paddle type). The dissolution test was performed using 900 ml of dissolution media as (pH 1.2, 4.0, 6.8 and 7.4) at 37 ± 0.5° and 100 rpm. The samples (5 mL) of the solution were withdrawn from the apparatus at 0, 5, 10, 15, 30min, 1, 2, 4, 6, 8, 12 and 24 h intervals. The samples were replaced with fresh dissolution media respectively. The samples were filtered and suitably diluted. Absorbance values of these solutions were measured against respective buffer solutions at 285, 306 and 320 nm using UV Spectrophotometer. The percentage drug release was calculated.

RESULTS AND DISCUSSION

Preformulation Study

Naringenin was identified and characterized as per the tests for identification given in official monograph. The physical appearance and melting point of the drug sample under investigation was found to be same as that of the official reports. U.V. estimation of Naringenin was done by U.V. spectrophotometric method. The calibration curve was prepared in methanol and different type of buffer. The data was regressed to obtain a straight line. The R^2 value was found to be 0.995 in methanol indicating good linearity. The calibration curve was found to obey Beer Lambert `s law in the concentration range studied.

The solubility of Naringenin was determined in organic solvents. It was found to be insoluble in water, freely soluble in methanol and other organic solvents.

Drug excipient compatibility

The I.R Spectrum of Naringenin (NAR), mannitoland optimizedformulations was taken and the characteristic peaks were compared with the I.R spectrum of the selected samples. The results of the I.R analysis revealed that no interaction between drug and carrier [Figure 1].

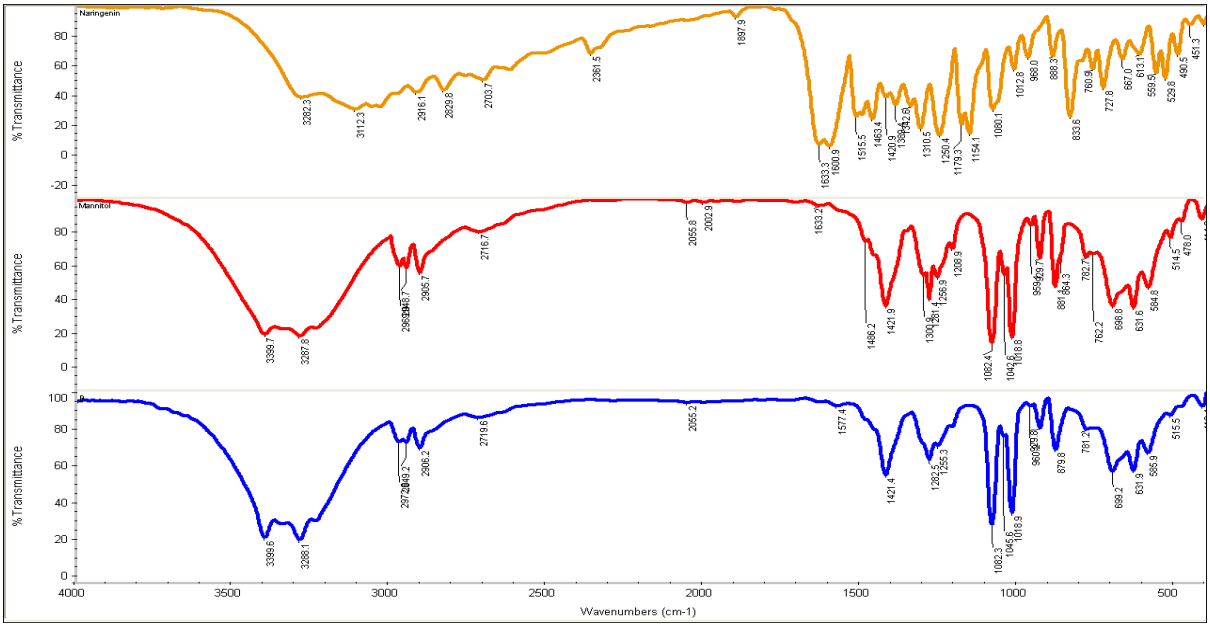

Figure 1: FTIR spectra of naringenin, mannitol and naringenin nanocrystals

Particle size, Polydispersity index (PDI) and zeta potential

The particle size and PDI of the NARNC formulation (F2) was found to be 349.2 nm and 0.237, respectively. The zeta potential of NARNC formulation was found to be 13.86 mV [Fig.2 (a) and Figure. 2 (b)].

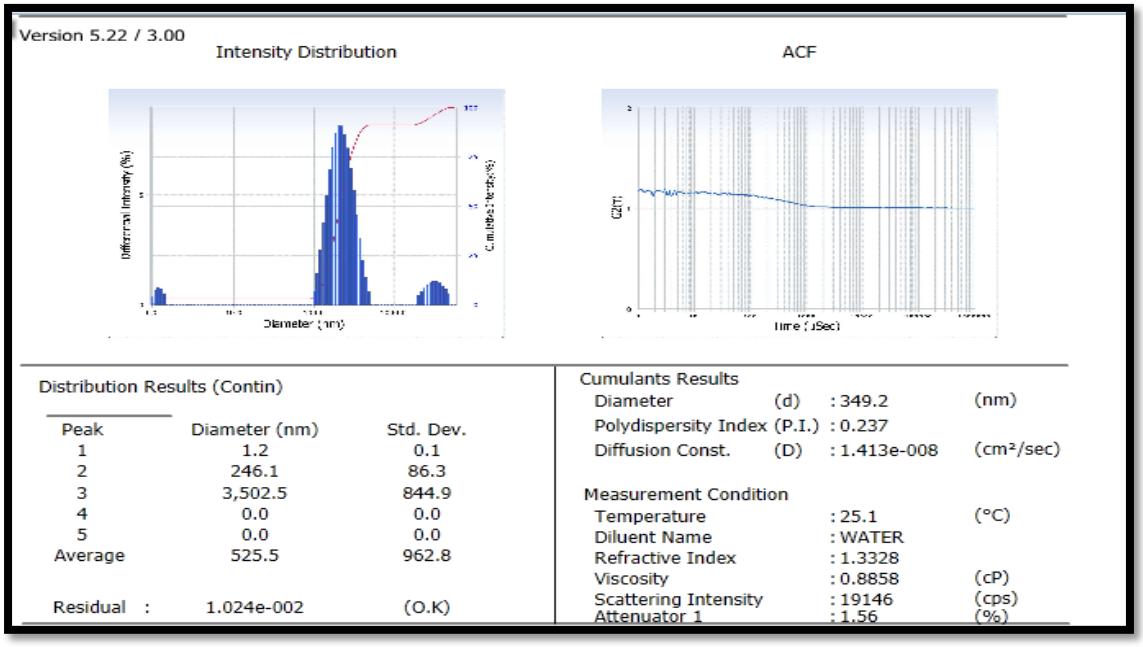

Figure 2: (a) Particle size of the optimized formulation of NC F2

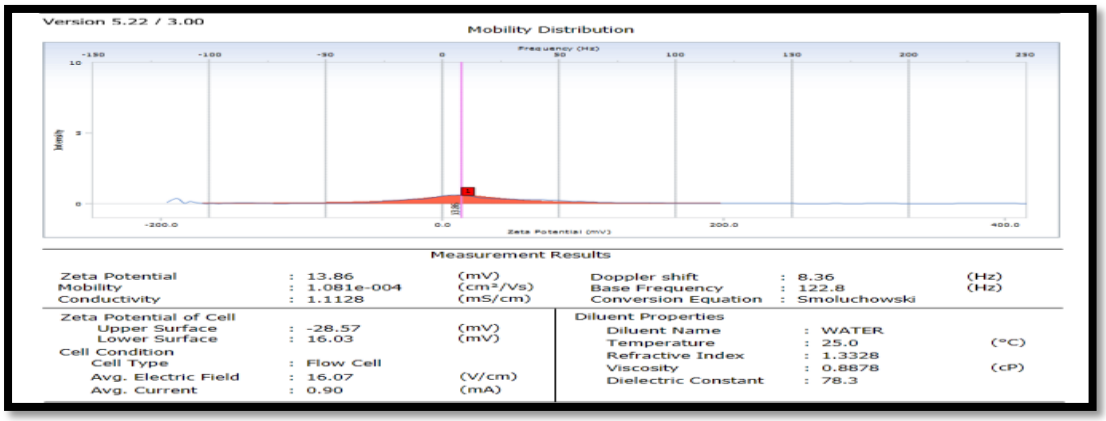

Figure. 2 (b) Zeta potential of the optimized formulation of NCF2

Drug Content and Entrapment Efficiency

Drug content and entrapment efficiency of the NARNC formulation f2 was found to be 92.34% and 88.16% respectively.

Shape and Surface Morphology

The morphological micrographs of naringenin nanocrystalss were examined by SEM as demonstrated in Fig.3. It was observed that the Naringenin nanocrystalss were rod-like shape with smooth surface. From the SEM result it was observed that the sample is crystalline in nature.

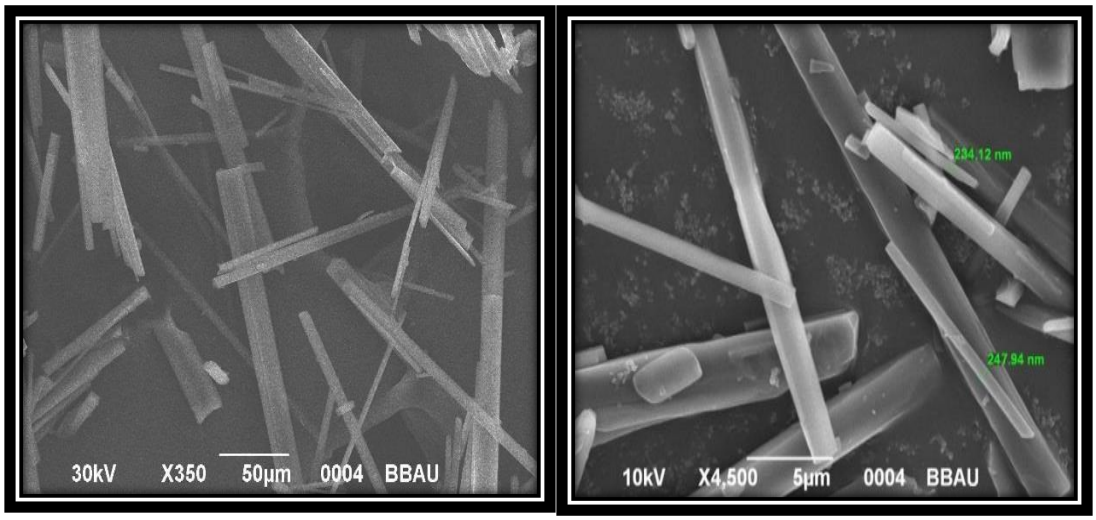

Figure 3: Scanning Electron Photomicrograph of NARNC formulation F2

Differential Scanning Calorimetry

DSC thermograms showed that the endothermic melting peak of naringenin nanocrystalss was long and slightly shifted when compared to that of the naringenin drug. These results indicated possibility of an alteration of crystallinity of drug to other crystalline forms or to amorphous stage and presence of mannitol bound to the surface of nanocrystals [Fig. 4 (a), 4 (b), and 4 (c)].

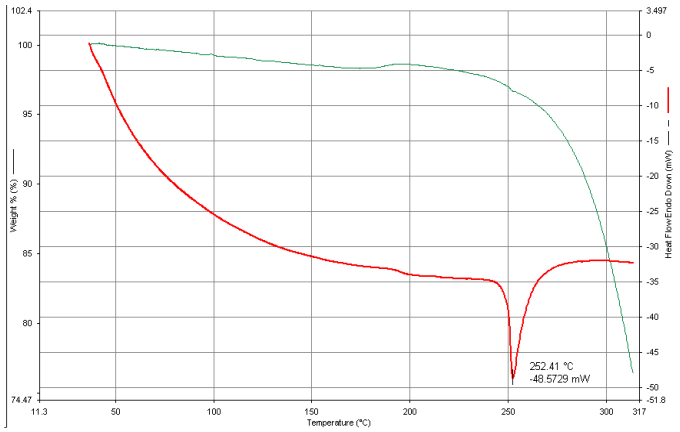

Figure 4: (a) DSC thermogram of Naringenin

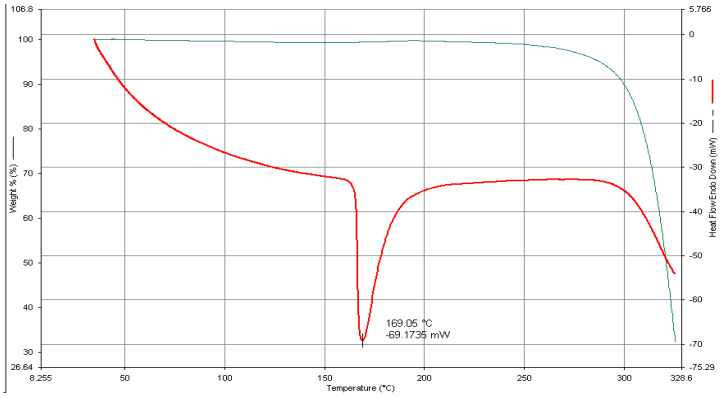

Figure 4: (b) DSC thermogram of Mannitol

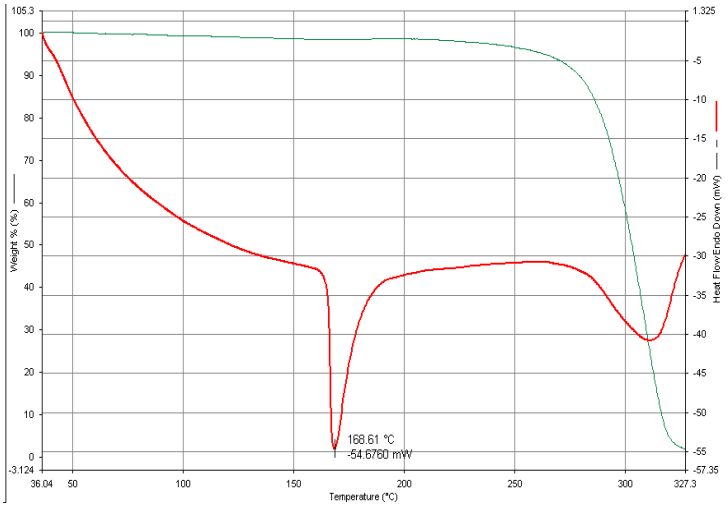

Figure 4 (c) DSC thermogram of Nanocrystals formulation (NC1)

X-Ray Diffraction

Powder X-ray diffraction patterns of Naringenin and Naringenin Nanocrystalss were also studied in order to gain insights into the crystallanity differences. The powder X-ray diffractograms are shown in Fig.5 (a), 5 (b) & 5 (c). Naringenin showed intrinsic peaks at the diffraction angles, indicates a typical crystalline pattern. The spectrum of nanocrystals showed that some peaks of

pure naringenin were absent and intensity of peaks was reduced. The result indicates that the drug in nanocrystals was amorphous as compared to the pure drug.

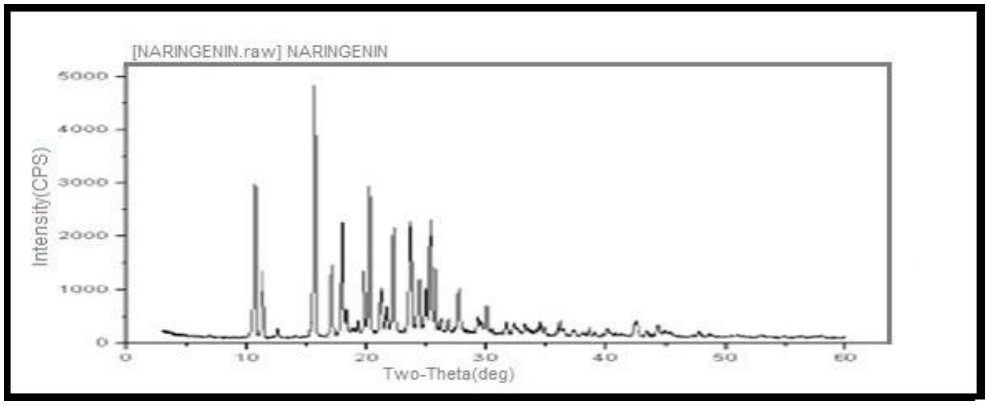

Figure. 5 (a) Powder X-ray diffraction pattern of Naringenin

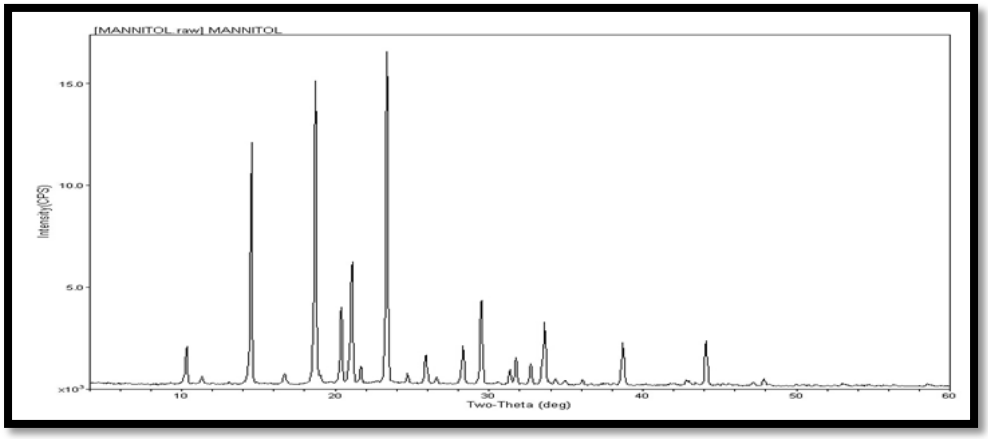

Figure. 5 (b) Powder X-ray diffraction pattern of Mannitol

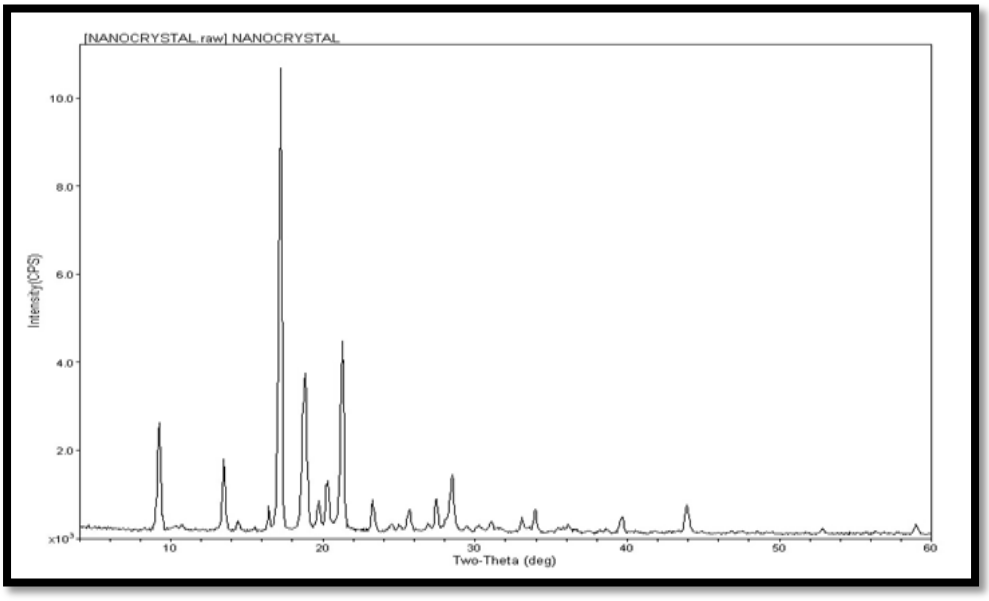

Figure 5 (c) Powder X-ray diffraction pattern of Nanocrystals formulation (F2)

Solubility analysis

In this study, kinetics of saturation solubility of Naringenin nanocrystals formulations were studied in the different dissolution media. It was found that naringenin solubility was pH-dependent. Higher solubility of naringenin from all samples was observed in distilled water pH 7.4 buffer as compared to that in other buffer. The formulation F2 showed higher saturation solubility in phosphate buffer pH 6.8 and 7.4. It shows poor solubility in acidic medium while exhibits excellent solubility in alkaline media [Table 2].

Table 2:. Solubility study of all formulation (F1-F4) in Different media

Batch code	Distilled Water (mg/mL)	pH 7.4 (mg/mL)	pH 6.8 (mg/mL)	pH 4.0 (mg/mL)	pH 1.2 (mg/mL)
F1	7.60±0.16	6.9±0.017	5.74±0.055	1.38±0.060	1.76±0.045
F2	8.78±0.03	7.79±0.025	7.38±0.055	2.19±0.060	1.90±0.015
F3	7.19±0.049	6.74±0.052	5.25±0.049	1.32±0.023	1.61±0.49
F4	6.47±0.085	6.40±0.075	4.92±0.045	1.26±0.060	1.07±0.015
API	0.012±0.011	0.023±0.0057	0.022±0.0057	0.013±0.0057	0.012±0.011

***In-Vitro* dissolutiuon study of all formulation**

Dissolution profiles of the pure Naringenin powder and nanocrystals formulations (F1-F4) in phosphate buffer pH 6.8 are shown in Table 3. In phosphate buffer pH 6.8, the dissolved formulation F1 increased as compared to that of the all formulation and naringenin powder. When compared with the all formulation and naringenin powder, nanocrystals formulations F1 had higher rate and control release. Formulation F1 showed better release to the other formulations.

Table 3: Percentage drug release of all formulation in pH 6.8

S. NO.	Time (hrs)	% Cumulative drug release of NC formulation				
		F1	F2	F3	F4	API
1.	0	0	0	0	0	0
2.	0.083	2.44±0.59	2.19±0.23	1.9±0.72	2.45±1.41	0.71±0.34
3.	0.16	4±0.58	3.26±0.52	2.46±0.88	3.40±0.41	1.67±0.21
4.	0.25	6.20±1.11	4.85±0.61	3.21±0.71	4.94±0.15	2.9±0.44
5.	0.5	7.81±1.62	7.90±0.47	4.13±0.65	8.56±1.05	7.69±1.37
6.	1	9.80±3.28	18.47±1.13	7.09±0.25	12.22±0.095	14.76±2.05
7.	2	18.26±1.19	28.27±1.66	14.82±2.02	21.56±0.25	21.56±2.03
8.	4	27.60±1.72	42.02±1.84	26.83±2.67	31.26±1.48	24.97±1.45
9.	6	33.20±2.08	53.25±2.27	32.57±1.94	36.40±0.072	30.9±0.98
10.	8	41.62±1.67	60.73±2.28	38.85±1.26	39.78±0.037	34.51±1.86
11.	12	53.47±3.88	71.41±2.31	45.54±1.87	44.13±0.58	38.48±1.11
12.	24	61.18±3.51	76.50±1.98	51.60±2.02	46.95±0.41	42.06±1.33

***In-Vitro* dissolution study of the optimized formulation**

Dissolution profiles of the pure naringenin powder and optimized nanocrystals formulations F2 in phosphate buffer pH 7.4, 6.8 and 1.2 are shown in Table 4 (a) & 4 (b). In phosphate buffer pH 7.4, the percent drug dissolved of the formulation F2 increased in control manner as compared to that of the Naringenin powder and other pH release.

Table 4: (a) Percentage drug release of the optimized formulation in different pH

S. NO.	Time (hrs)	% Drug release pH 7.4 F2	pH 7.4 API	pH 6.8 F2	pH 6.8 API
1.	0	0	0	0	0
2.	0.083	2.32±0.10	2.16±1.41	2.88±0.32	2.39±0.34
3.	0.167	3.43±0.09	3.46±0.23	4.44±1.05	4.38±0.10
4.	0.25	5.18±0.17	5.36±0.32	8.48±1.72	7.12±1.08
5.	0.5	7.69±0.22	8.02±1.08	12.33±0.46	11.62±0.14
6.	1	20.36±0.39	12.6±0.72	19.61±0.80	25.16±1.14
7.	2	36.43±0.06	19.77±1.36	27.65±2.22	29.72±0.32
8.	4	54.07±0.09	23.36±0.25	39.04±3.41	30.18±2.39
9.	6	62.82±0.14	37.89±0.13	47.99±2.63	34.43±0.09
10.	8	69.3±0.27	41.17±0.32	53.8±2.39	42.19±0.25
11.	12	74.37±0.22	49.23±0.92	61.24±3.13	47.39±2.63

Table 4: (b) Percentage drug release of the optimized formulation in different pH

S. NO.	Time (min)	% Drug release pH 4.0 F2	pH 4.0 API	pH 1.2 F2	pH 1.2 API
1.	0	0	0	0	0
2.	0.083	0.85±0.13	0.39±1.05	0.75±0.32	0.46±0.59
3.	0.167	1.21±0.35	0.96±1.72	1.77±0.22	1.26±0.095
4.	0.25	3.30±0.25	2.36±0.32	2.28±0.47	2.36±1.11
5.	0.5	5.92±0.23	5.03±0.25	3.77±0.19	5.02±0.44
6.	1	9.57±0.64	7.86±2.63	7.39±0.33	9.6±1.41
7.	2	14.13±0.47	9.36±0.13	12.53±0.77	13.73±2.02
8.	4	23.08±0.13	12.7±0.39	17.3±0.63	19.42±1.94
9.	6	30.00±0.77	22.3±0.09	24.62±0.72	22.36±0.21
10.	8	36.53±1.07	29.58±3.13	30.05±1.19	29.98±0.68
11.	12	42.17±1.08	31.72±2.03	37.25±2.67	33.53±1.07
12.	24	46.67±0.46	34.6±0.92	46.89±1.37	38.7±0.60

Release Kinetics Study

It is well documented that drug release from carrier shows a typical time-dependent profile (i.e. increased drug release with time because of increased diffusion path length). The release mechanism of Naringenin from optimized formulation (F2) was determined by comparing their respective correlation coefficient. The release pattern was fit into korsmeyer peppas model and r^2 value was found to be 0.9558 [Table 5].

Table 5:. Kinetic study of the Release Data of Naringenin formulation F2

Formulation	R^2 Zero order		First order		Koresmeyer Pappas Model		Higuchi	
F1	R^2	K_O	R^2	K_1	R^2	K_n	R^2	k_t
	0.6755	3.2675	0.677	4.3628	0.9558	1.1774	0.7212	8.855

In-Vivo Study

Pharmacokinetic Study

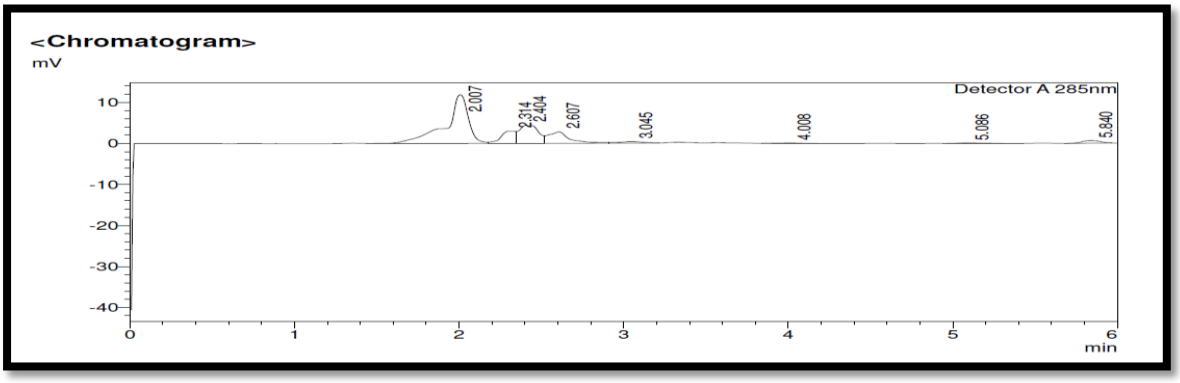

Figure. 6 (a) HPLC chromatogram of blank serum

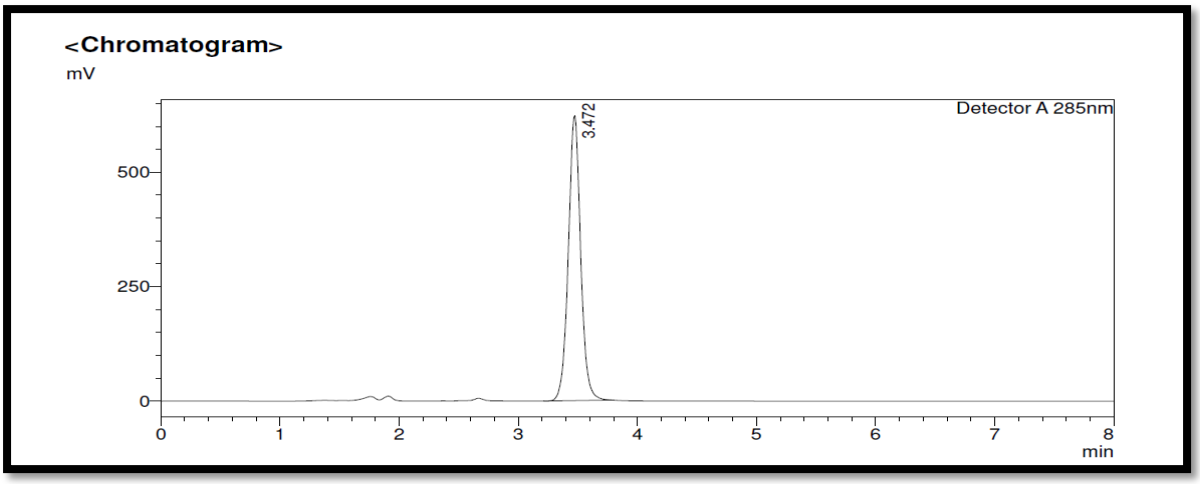

Figure. 6 (b) HPLC chromatogram of naringenin

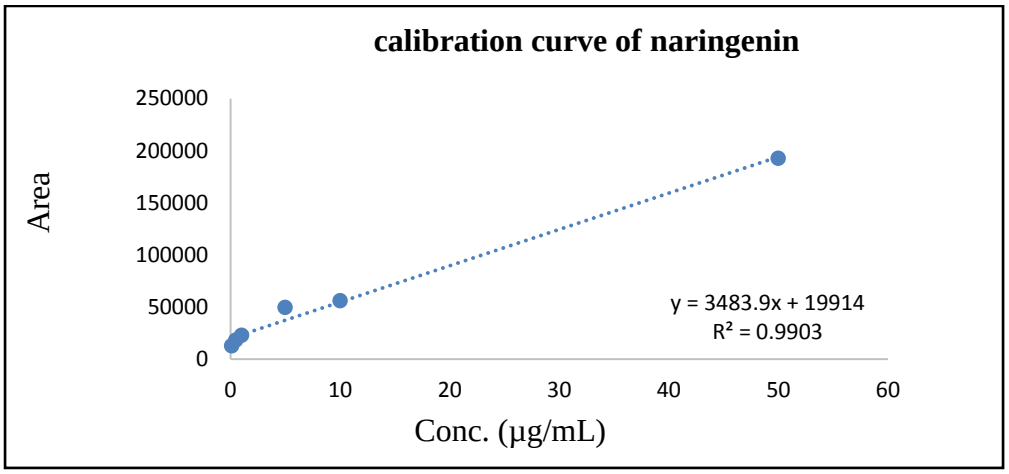

Figure.: 7. Standard plot of Naringenin in ACN using HPLC

Table 6: Pharmacokinetic parameters of NCF2 in rats

Pharmacokinetic parameter	Standard drug	Formulation
C_{max} (ng/mL)	5.67	7.77
T_{max} (h)	4	4
$t_{1/2}$(h)	4.5787	9.2849
$AUC_{(0-\infty)}$ (ng.h/mL)	41.6755	110.2403
$AUMC_{(0-\infty)}$ (ng.h^2/mL)	350.0032	1672.2857
MRT (h)	8.3983	15.1695

From HPLC data, we observed that both API and NC has T_{max} of 4 h. However, NC had better bioavailability (7.77 ng/mL) than parent API (5.67 ng/mL). This observation suggested that NC form of Naringenin have better bioavailability than API. From our result, it was also observed that mean residence time (MRT) was also increased for NC which signified better bioavailability also.

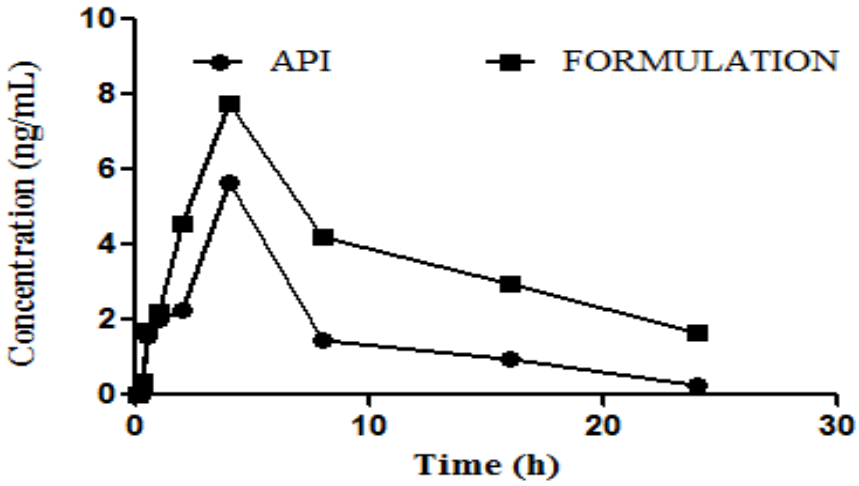

Figure 8: Pharmacokinetic profile of naringenin (API) and formulation (NNC) in rats

Stability Studies

The optimized NC formulation was stored in glass vial with rubber stopper at room temperature (37±1^{0}C), refrigerator (4±1^{0}C)and stability chamber (45±1^{0}C) for three month. The samples were withdrawn at regular intervals and were analysed for % drug content. [Table 6].

Table 7: (a). Degradation of Optimized Formulation F2 batch

S. No.	Sampling Interval (in days)	% Drug Remaining			Physical Appearance		
		37±1˚C	45±1ºC	4±1˚C	37±1˚C	45±1ºC	4±1˚C
1.	0	92.30±0.16	92.30±0.16	92.48±0.01	+	+	+
2.	15	91.51±0.14	90.42±0.21	90.3±0.15	+	+	+
3.	30	89.96±0.13	88.31±0.16	88.44±0.30	+	+	+
4.	45	88.29±0.14	86.89±0.11	86.60±0.33	+	+	+
5.	60	86.42±0.13	85.28±0.09	85.24±0.11	+	+	+
6.	75	85.83±0.09	83.40±0.16	84.46±0.11	+	+	+
7.	90	83.73±0.08	81.67±0.20	81.99±0.20	+	+	+

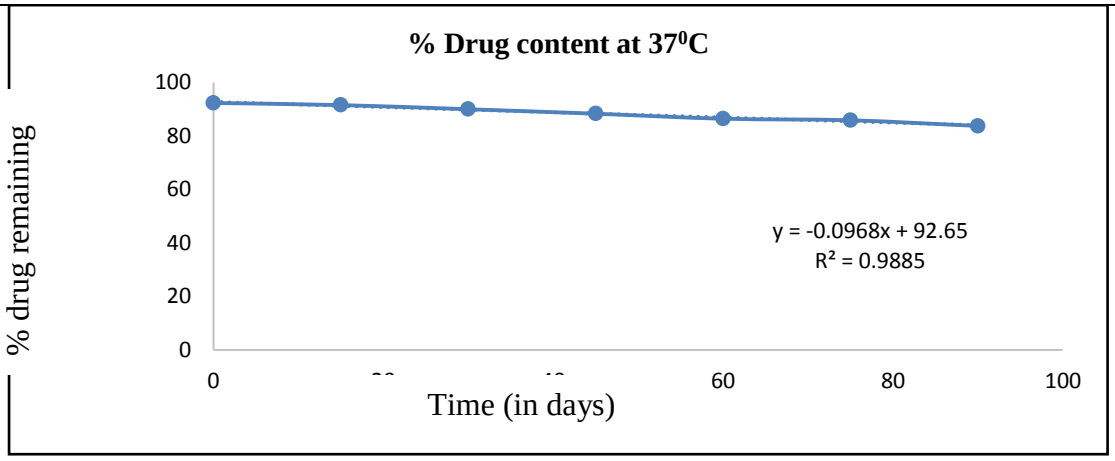

Figure. 9 (a). Degradation Kinetics of Naringenin from NC at room temp. (37±1^{0}C)

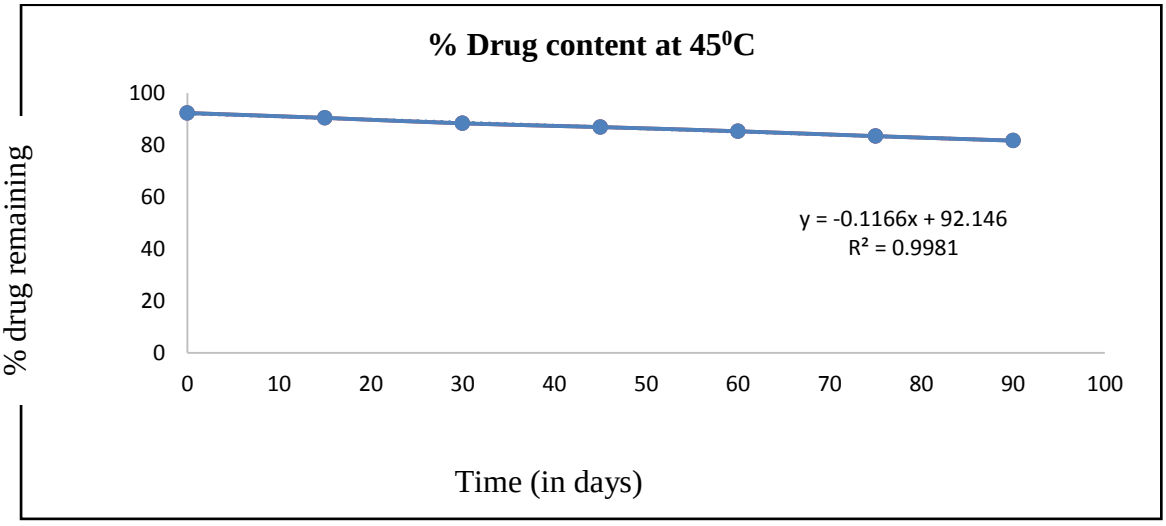

Figure. 9 (b). Degradation Kinetics of Naringenin from NC at stability chamber (45±1^{0}C)

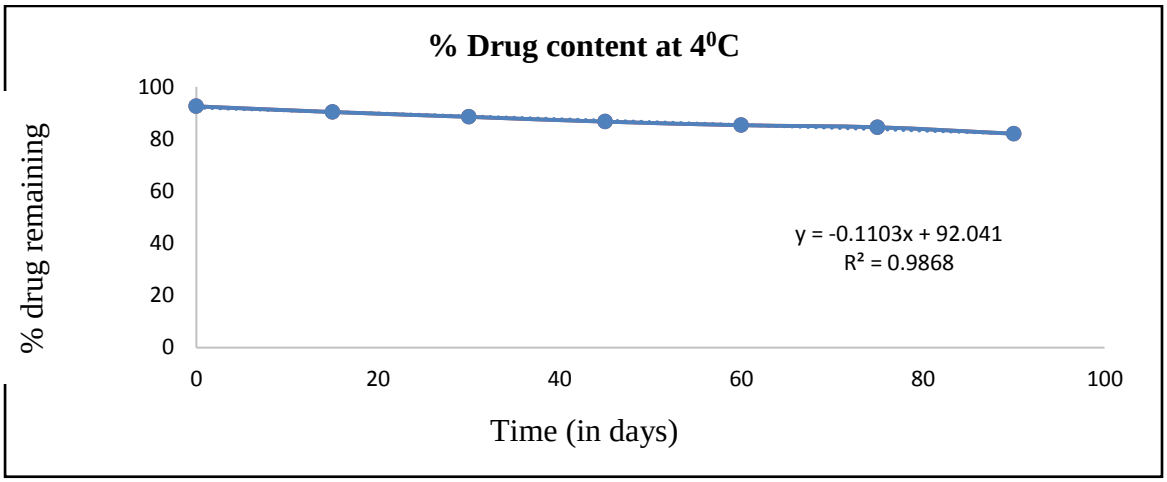

Figure. 9 (c) Degradation Kinetics of Naringenin from NC under refrigeration (4±1⁰C)

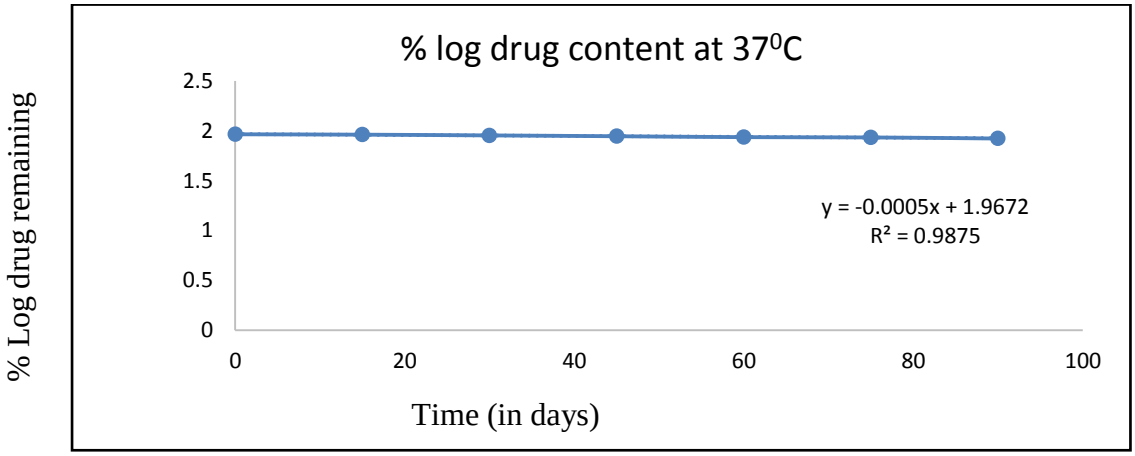

Figure. 9 (d) Degradation of % log drug release with time at room temperature (37˚C±1ºC)

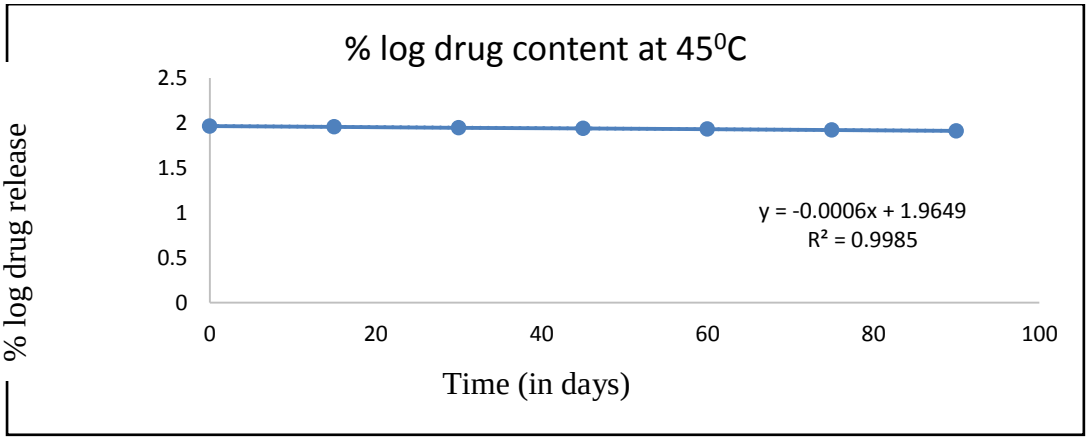

Figure. 9 (e) Degradation of % log drug release with time in stability chamber (45˚C±1ºC)

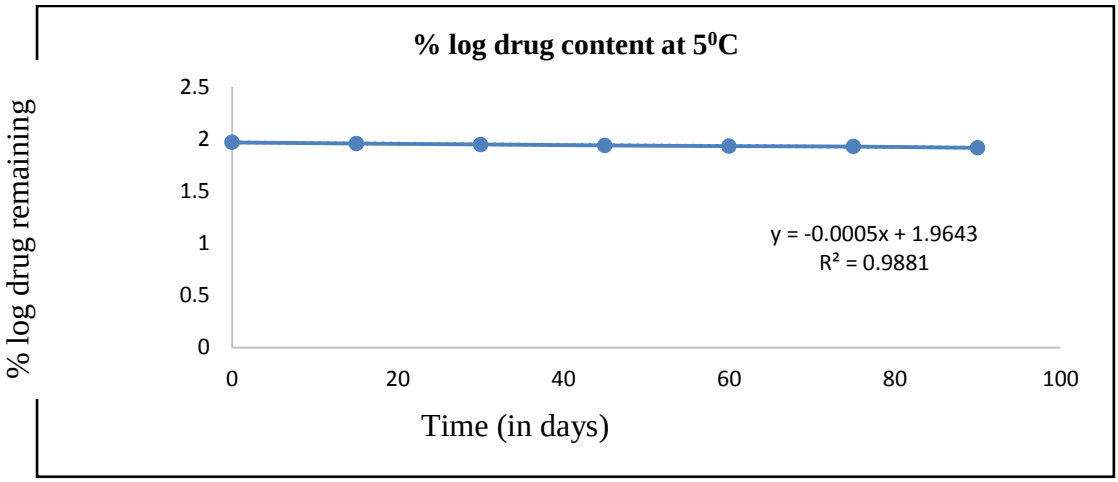

Figure. 9 (f) Degradation of % log drug release with time under refrigeration (4˚C±1ºC)

Table 7 (b):. Shelf-life, half-life of the optimized formulation (F2)

S. No.	Parameters	At 37 ^{0}C	At 4 ^{0}C	At 45 ^{0}C
1.	K(day^{-1})	1.07x10^{-3}	1.32x10^{-3}	1.35x10^{-3}
2.	$t_{1/2}$ (days)	647.66	521.83	513.33
3.	T_{10}% (days)	97.19	78.78	77.03

The optimized NC was stored in glass vial with rubber stopper and hard gelatin capsule at room temperature (37^{0}C±1^{0}C), refrigerator (4^{0}C±1^{0}C) and stability chamber (45±1^{0}C) for three months, there was no apparent change in the physical parameters, good stability of the developed NC formulation at room temperature (37^{0}C±1^{0}C). Furthermore, there was no significant difference in percentage drug remaining. It was also seen that the formulation was compatible with the hard gelatin capsule shells, as there was no sign of capsule shell deformation, drug precipitation or capsule leaks.

Drug content of F2 was slightly decreased with time at room temperature (37^{0}C±1^{0}C) but decreased with high rate at (4^{0}C±1^{0}C) and (45^{0}C ±1^{0}C).

CONCLUSION

These studies confirmed the stability study of the developed naringenin nanocrystalss formulation can be used for long time and can be kept at room temperature and ambient condition. Finally, we concluded that nanocrystals form of Naringenin had better bioavailability and good solubility, which might be beneficial for future formulation design perspective.

ACKNOWLEDGEMENT

The authors are thankful to MP LLC for providing the samples. The authors are thankful to the Department of Pharmaceutical Technology, Babasaheb Bhimrao Ambedkar University, Lucknow

and Dr. B. R. Ambedkar Pooja College of Pharmacy, Gorakhpur, India, who graciously supported us.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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