OriginalArticle

Implementation Of Box Behnken Design To Fabricate The Formulation, Optimization And Dissolution Of Blueberry Nanosuspension

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Abstract

The present study aims to enhance the bioavailability of Blueberry by formulating it as nanosuspension. Blueberry mainly consists of anthocyanins which have strong antihyperlipidemic activity but suffers from low bioavailability which causes hindrances in its therapeutic activity to treat hyperlipidemia. Hence to improve its bioavailability, nanosuspension of blueberry was prepared. Box-Behnken design was applied to predict the effect of independent variables namely concentration of chitosan and concentration of tripolyphosphate (TPP) and stirring speed on two responses namely particle size and entrapment efficiency. The design suggested that a formulation having a particle size of 205.05nm and entrapment efficiency of 76.60 % will be an optimized formulation if it has chitosan, TPP,and stirring speed of 0.5%, 0.05%,and 1050 rpm respectively. The formulation suggested by design was prepared and it was found that particle size and entrapment efficiency of nanosuspension 198.5 nm and 75.14±0.56 % respectively. In addition, it was found to have polydispersity index, refractive index, zeta potential of 0.351, 1.33, 21.08mv respectively.SEM analysis showed the droplet size under 200nm.When compared towards blueberry nanosuspension with drug extract release it shows the controlled release of drug up to 8 hrs which enhances the bioavailability of drug and drug extract suspension showed the maximum release in 2hrs which shows the immediate release of the drug. As a result, our research shows that nanotechnology might be utilized as an effective approach increasing the bioavailability of blueberry nanosuspension and this article also focuses on the antihyperlipidemic activity of anthocyanin.

Keywords: Blueberry, Anthocyanin, Nanosuspension, Optimization, Dissolution

Abberivations:

ANOVA: Analysis of variance
AUC: Area under the curve
PPAR: Peroxisome proliferator-activated receptor.
CCD: Central composite design
CV: Coefficient of variation
FTIR: Fourier transforms infrared spectroscopy
HPMC: Hydroxypropyl methylcellulose
PDI: Polydispersity index
RSM: Response surface methodology
SEM: Scanning electron microscopy
TPP: Sodium tripolyphosphate

1. INTRODUCTION

1.1 Hyperlipidemia

The term "hyperlipidemia" refers to a group of inherited and acquired illnesses that are characterized by high lipid levels in the body. It contributes to several metabolic risk factors, like coronary heart disease, dyslipidemia, type II diabetes, gastrointestinal, reproductive, and hypertension. Around 19.1 million deaths worldwide in 2020 were attributed to cardiovascular diseases. The death rate, adjusted for age, was 239.8 per 100,000 people. The prevalence rate, adjusted for age, was 7354.1 per 100,000[1]. When it was first introduced in the 1960s, the Frederickson (World Health) categorization of dyslipidemias had 5 categories. As per the report published in 2020, low-density lipoprotein cholesterol accounted for 4.5 million death[2]. Hyperlipidemia can be controlled by changing their diet and lifestyle to some extent but there are some medications also available to control hyperlipidemia namely statins (atorvastatin, lovastatin, simvastatin)[3]. These medications stop the production of cholesterol by the liver[4]. But these medications are associated with various side effects and the development of treatment resistance which limits their use in hyperlipidemia. The prevalence of medicinal herbs in the treatment of several diseases is inclining continuously but their use is also limited due to various limitations like

poor aqueous solubility, and low bioavailability which must be overcome for their effective use. These limitations can be combated by formulating herbal drugs in the novel delivery system like nanosuspension, nanoemulsion, solid lipid nanoparticles, polymeric micelles, dendrimers, etc[5,6]. Modern phytopharmaceutical research resolves the various scientific issues (such as the determination of pharmacokinetics, mechanism of action, and accuracy in doses). Blueberry is also a herbal drug related to this category but the problem is associated with it low bioavailability due to low permeability but it shows excellent in vitro antioxidant potential of their rich polyphenolic components, blueberries were first hailed as a super fruit. However,due to their low bioavailability, direct antioxidant action seems implausible[7]. The method by which a drug is delivered can have a significant effect on efficacy. Some drug has an optimum concentration range within which maximum benefits are achieved and concentration below or above this range can be toxic and do not show any benefit at all. As per data given in research literature, it was found that over 133,000 men, as well as women followed for over 24 years, found that the highest intake of blueberries was related to the least gaining weight (0.64 kg over 4 years) in a comparison of intakes of 16 popular fruits. In a study of 124,000 people, the highest link between higher anthocyanin consumption and decreased weight gain (0.1 kg/10 mg anthocyanins) was found among the six groups of flavonoids[8]. There was little knowledge of the processes through which anthocyanin exerts its anti-obesity properties[9]. Consuming blueberries also decreased the amount of belly fat, improved PPAR activity in adipose tissue and skeletal muscle, and altered PPAR transcripts that are important in fat oxidation as well as glucose uptake/oxidation. Anthocyanins, which are present in blueberries, can change the activity of PPARs, which control the metabolism of energy substrates[10]. Medication delivery strategies have been developed using nanotechnology to address the problems with current conventional drug administration systems. Anthocyanin stability improvement is a significant issue that requires immediate attention. Finding a method to get beyond this barrier of anthocyanin's unstable, which could result in inadequate bioavailability, is a significant problem for food applications. To increase bioavailability before ingestion and during digestion, several formulations such as protein complexes, microencapsulation, and nanoparticulate systems may be used[11]. Additionally, nanosuspension can be freeze-dried or spray-dried, and their nanoparticles can be a part of a solid matrix[12].Greek term nano means "dwarf" in English. It shows that passive targeting is the preferred gathering of chemotherapeutic agent in solid tumors as a result which enhance the vascular permeability of tumor tissues compared with healthy tissues. Drug stability and bioavailability can be increased by the use of nanosuspension technology[13].Stabilizers, organic solvents, and other additives including buffers, salts, polyols, cosmogenic, and cryoprotectants the ability to make nanosuspension[14].

2. MATERIAL AND METHODS

2.1 Material

Blueberry was purchased from Kshipra Biotech, Indore, India. Chitosan was provided as a gift sample from Kshipra Biotech, Indore, India. Sodium Tripolyphosphate was purchased from Merck, Mumbai, India. Other chemicals were purchased of analytical grade.

2.2 Determination of anthocyanin content in blueberries

200g of blueberries was taken and blended to provide a soft puree. The extraction of soft puree was performed using ethanol and 0. 1 M HCl in ratio of 85:15%, V/V. The puree and solvent for extraction was taken in ratio of 1:2 and mixing was carried in a magnetic stirrer for at least 1 hour followed by filtration using a Buchner funnel to collect the supernatant solution (test sample). This procedure was done in triplicate. Then test samples of 10ml were diluted with pH 1.0 buffer and pH 4.5 buffer in 50ml volumetric flask[15]. Then absorbance of both test samples was determined at 520nm by using UV spectrophotometer (Shimadzu, UV-1800, Japan) followed by calculation of anthocyanin content using the equation 1.

$$\text{Anthocyanin pigment} = \frac{A.\,MW.\,DF.\,10^3}{\varepsilon.l}$$

A=Absorbance
MW=Molecular weight
DF=Dilution factor
L=Path length (cm)
ε = Molar extinction coefficient
10^3= Factor for conversion of g to mg

2.3 Method of preparation for Nanosuspension

Nanosuspension of blueberry was formulated using the ionic gelation method in which an optimized quantity of chitosan was dissolved in acetic acid solution followed by addition of fresh extract of blueberry. The pH of the solution was maintained at 6.5 and then solution of sodium tripolyphosphate at a concentration of 1mg/ml was added dropwise. The resulting solution was sonicated and subjected to a centrifuge at 4˚C for 15 min. The supernatant was collected, formed pellets were then redispersed to analyze for size and other parameters[16].

2.4 Optimization of Nanosuspension by Box-Behnken Design

Various crucial formulation variables required to prepare nanosuspension were optimized by means of the Box-Behnken design using Design expert®,version 10.0.4, Stat-Ease, Minneapolis, USA. In this, chitosan concentration (X1), sodium

tripolyphosphate concentration (X2) and (X3) at different levels were employed as independent variables whilst particle size (Y1) and entrapment efficiency (Y2) were used as dependant variables Table 1.The design suggested a total 15 randomized formulations which were prepared and performed for evaluation of particle size and entrapment efficiency which are crucial parameters to formulate nanosuspension[17,18].

Table 1: Levels of independent variables along with their dependent variables

Independent variables			
Levels	**Chitosan concentration (%)**	**TPP Concentration (%)**	**Stirring speed(seconds)**
Low (-1)	0. 25	0. 01	600
Medium (0)	0. 50	0. 05	1050
High (+1)	0. 75	0. 1	1500
Dependent variables			
Particle size Entrapment efficiency			

3. CHARACTERIZATION OF OPTIMIZED NANOSUSPENSION

3.1 Particle Size, Zeta Potential and Polydispersity Index

The particle size is an important characteristic of nanosuspension since smaller particles provides greater surface area and greater surface area provides improvement in absorption of the drug. The particle size,zeta potential and polydispersity index of the optimized nanosuspension formulation was calculated by Malvern Zetasizer (Zetasizer 1000 HAS, Malvern Instruments, UK). For this, formulation was diluted ten times with distilled water followed by placing in a cuvette for calculation using the angle of 90° at room temperature[19].

3.2 pH and refractive index determination

Optimized formulation was evaluated for determination of pH at 25±0.5°C employing a calibrated pH meter (Ajanta industries, pvt. Ltd).The Refractive index was calculated using an Abbe refractometer (Nirmal International, India)[20].

3.3 Entrapment efficiency

Prepared formulation of nanosuspension was centrifuged using cooling ultracentrifuge for 20 minutes at 4°C employing speed of 20, 000 rpm[21]. The supernatant was collected, diluted and analyzed using UV spectrophotometer to determine the amount of free drug using equation 2.

$$\textbf{Drug entrapment efficiency} = \frac{\textbf{Experimental drug content} \times 100}{\textbf{Theoretical drug content}}$$

3.4 Assessment of surface morphology using scanning electron microscopy (SEM)

The surface morphology of the optimized formulation was assessed using scanning electron microscopy (SEM). For this, the solid nanoparticles (obtained by using a hot plate to evaporate excess solvent) was used. Using a scanning electron microscope with a secondary electron detector (JEOL,JSM-6400, Japan), digital pictures were obtained at a 15kv applied potential[22].

3.5 Assessment of surface morphology using transmission electron microscopy (TEM)

The surface morphology of the optimized formulation was assessed using a Morgagni 268D transmission electron microscopy TEM (FEI, Hillsbro, Holland) running at 70 kV.A drop of nanosuspension was taken and deposited of 2% (w/v) phosphotungstic acid was used to further stain a wax paper and air dried. The slide was observed for surface morphology with TEM[23].

3.6 Assessment of interactions between drug and excipients employing Fourier Transform Infrared Spectroscopy (FTIR)

Potassium bromide pellet technique was employed for FTIR analysis of the drug, chitosan, TPP, In this technique, a homogenous mixture was developed by adding the sample of drug and dried form of optimized formulation separately with potassium bromide in the ratio of 1:10 followed by grinding the mixture. Then a small amount of powdered mixture was compressed to a thin semitransparent film (pellets) by applications of pressure[24]. The pellets were analyzed for IR spectrum within the range of 500-400cm^{-1} using the FTIR spectrophotometer (Shimadzu, 8400S, Japan) [25].

3.7 In -vitro Dissolution Studies of Optimized Nanosuspension

These studies were performed to investigate the in-vitro dissolving behavior of optimized nanosuspension in comparison to coarse plant extract. Coarse herbal extracts and nanosuspension were tested for in vitro dissolving using a dialysis bag.

In this study, one milliliter of optimized nanosuspension and plant extract were placed into the dialysis bag followed by dipping into the beaker with 80ml of phosphate buffer of pH 7.4. The beaker was placed over the magnetic stirrer having speed of 50 rpm and temperature was maintained at 37± 0.5°C for whole experimentation [26]. At periodical intervals (0, 15, 30, 45, 60, 75, 90,120, 240, 260, 480 min), an aliquot (5 ml) of the sample was removed and fresh buffer was added to maintain sink condition. The collected samples were analyzed using UV spectrophotometer at maximum wavelength of 523 nm[27].

3.8 Drug release models

To understand the release pattern of drug from a formulation several kinetic models are available which includes Higuchi model, Hixson-Crowell cube root law, Korsmeyer and peppas, zero- order and first -order kinetic model. These models were applied to the in vitro drug release data of optimized formulation using Fitter software to determine the best fitted model [28].

3. 9 Stability Studies

Over the course of three months, the physical stability of an optimized Nanosuspension was assessed at two different temperatures of 4℃ (in the refrigerator) and 25–30℃ (at room temperature) followed by analysis for particle size, entrapment efficiency, physical appearance and polydispersity index.

4. RESULT AND DISCUSSION

4.1 Determination of anthocyanin content in blueberries

Two types of anthocyanin were found in the blueberry namely monomeric anthocyanin and malvidin-3-glucoside anthocyanin in concentration of 23.72g/kg and 18.77g/kg respectively which demonstrate that anthocyanin is major component of blueberry which is responsible for antihyperlipidemic activity of blueberry. Ionic gelation methodology was adopted for the current study's nanosuspension preparation because it is straightforward, repeatable, and affordable. Important process variables like stabilizer and stabilizer quantity were improved for the creation of stable nanosuspension.

4. 2 Optimization of nanosuspension

The main formulation variables namely Chitosan and TPP were optimized using Box Behneken design which suggested 15 different formulations which were prepared by ionic gelation method. the results for particle size and percentage entrapment efficiency were found in range of 185nm-767nm and 63.4 to 81.2% respectively. The values for particle size and entrapment efficiency obtained after experimentation along with predicted value by design for these 15 formulations has been given in the Table 2. The optimization software proposed polynomial quadratic ($p \leq 0.0001$) for particle size (Y1) and linear equation (P= 0.001) for entrapment efficiency (Y2) when observed data for the dependent variables were submitted into it. The predicted R^2 value for both dependent variables was in agreement with the adjusted values due to a difference of less than 0. 2 which has been summarized in given Table 3 along with the mean, standard deviation, coefficient of variation(%) for all dependent variables. The quantitative effects for all independent variables used at different levels were predicted on both dependent variables. The software provided following polynomial equations for each dependent variable (response):

Particle size = +193.07+152.38 A -87.13 B +34.75C -11.75AB -48.00 AC+20.00 BC +255.84 Eq. 3

Entrapment efficiency= +72.71+5.91 A +2.74 B + 0.4000 C Eq. 4

Equation.3 showed the impact of chitosan concentration (A), Tripolyphosphate (TPP) concentration (B) and stirring speed (C) on the particle size of the formulation. The chitosan concentration possessed a positive impact, TPP concentration showed a negative impact and stirring speed showed a positive impact on particle size. This means that on increasing the chitosan concentration and the particle size will increase and on increasing the TPP, the particle size will be decreased. The combination of chitosan concentration and TPP concentration (AB) possessed a negative effect on particle size and the combination of chitosan and stirring speed (AC)also possessed a negative effect and the combination of TPP concentration and stirring speed possessed a positive effect on particle size which is also supported by 3D surface plot Figure.1 to Figure.3. ANOVA analysis and model summary statistics for experimental results provided a greater R^2 of 0.9853 for quadratic equation 3 and equation 4 and Figure 4 displayed the effect of chitosan concentration, TPP concentration, and stirring speed on entrapment efficiency [29]. All the independent variables showed a positive impact on entrapment efficiency which means that increase in concentration of chitosan, TPP and stirring speed will increase the entrapment efficiency. ANOVA analysis and model summary statistics for experimental result showed that greater of R^2 0.9857 for linear equation 4. The main objective of the Box-Behnken design was to obtain the optimal formulation components to prepare the nanosuspension. On the basis of experimentation and predictive results by the software, the design suggested that optimized formulation must have chitosan concentration, TPP concentration, stirring speed of 0.5%, 0.055 %, 1050 seconds respectively to obtain the best results of 198.5nm, 72.71% for particle size and entrapment efficiency respectively. Then the formulation suggested by design was prepared and found that the results of the dependent variables were very close to the results suggested by design Table 4.

Table 2 : Experimentation and predictive results of formulations suggested by design

Formulation	Independent variables			Dependent variables			
				Observed responses		Predicted responses	
Code	Chitosan Concentration%	TPP Concentration %	Stirring speed(sec)	Particle size (nm)Y1	Entrapment efficiency Y2	Particle size Y1	Entrapment efficiency Y2
FA1	0. 5	0. 1	600	225. 00	77. 40	234. 63	75. 05
FA2	0. 75	0. 1	1050	555. 00	76. 30	565. 25	81. 36
FA3	0. 75	0. 055	600	755. 00	75. 70	735. 13	78. 23
FA4	0. 25	0. 055	1500	480. 00	64. 50	499. 88	67. 20
FA5	0. 5	0. 055	1050	198. 50	72. 17	193. 07	72. 71
FA6	0. 75	0. 055	1500	695. 00	78. 20	708. 63	79. 03
FA7	0. 5	0. 055	1050	195. 50	76. 80	193. 07	72. 71
FA8	0. 75	0. 01	1050	767. 00	81. 10	763. 00	75. 89
FA9	0. 5	0. 055	1050	185. 20	74. 60	193. 07	72. 71
FA10	0. 25	0. 1	1050	280. 00	70. 80	284. 00	69. 54
FA11	0. 5	0. 01	1500	488. 00	68. 50	478. 38	70. 38
FA12	0. 25	0. 01	1050	445. 00	63. 40	434. 75	64. 06
FA13	0. 25	0. 055	600	348. 00	65. 30	334. 38	66. 40
FA14	0. 5	0. 01	600	425. 00	67. 70	448. 88	69. 58
FA15	0. 5	0. 1	1500	368. 00	78. 10	344. 13	75. 85

Table 3: Summarized regression analysis data for selected dependent variables

Quadratic model	R^2 value (coefficient of correlation)	Adjusted R^2	Predicted R^2	Adeq. Precision	Standard deviation	Mean	Coefficient of variation %(c. v. %)
Y1	0. 9949	0. 9857	0. 9209	29. 3605	23. 77	427. 35	5. 56
Y2	0. 7587	0. 6928	0. 5283	10. 6697	3. 14	72. 71	4. 32

Table 4: Predicted optimized formulation by design along with experimental results

Batch	Independent variables			Dependent variables	
	A%	B%	C%	Y1	Y2
Predicted	0. 5	0. 055	1050	193. 07	72. 71
Observed	0. 5	0. 055	1050	198. 50	72. 17

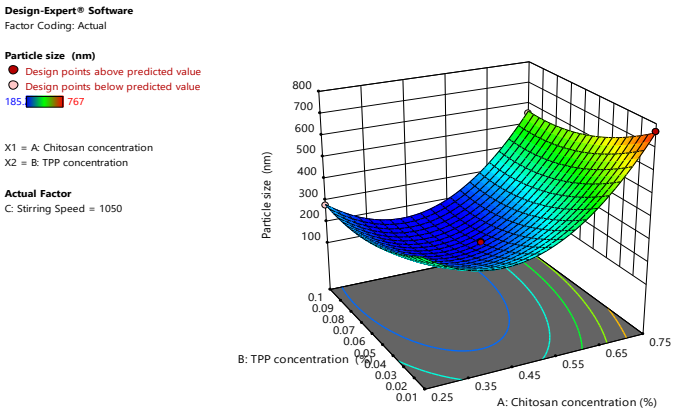

Figure 1: Effect of chitosan and TPP concentration on particle size shown in response surface counterplot

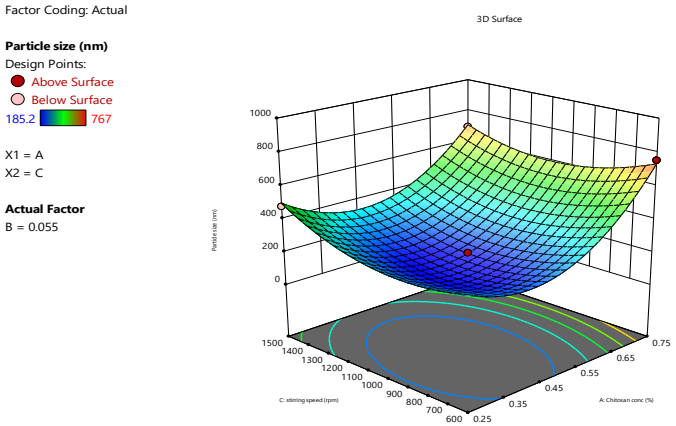

Figure 2: Effect of chitosan and stirring speed on particle size shown in response surface counterplot

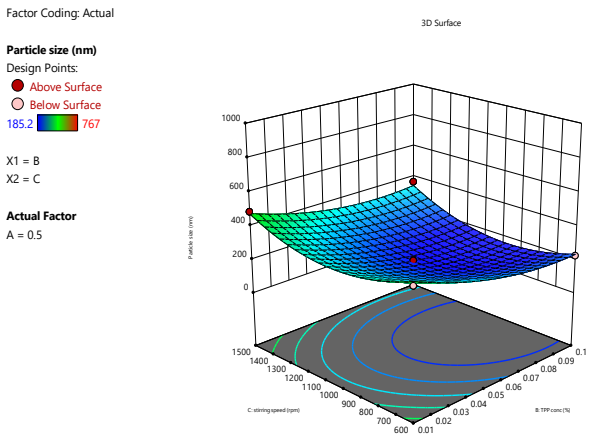

Figure 3: Effect of Stirring speed and TPP concentration on particle size shown in response surface counterplot

4.3 Entrapment Efficiency

Entrapment Efficiency increases when the coefficient of the X1-chitosan fixation in the mathematical statement is positive. Positive estimation of the X2-Speed coefficient indicates an increase in Y1, or Entanglement Efficiency. It proves the surface reaction's linearity and the shape plot's appearance in the Figure 4. The full model was found to be irrelevant, so areduced model was linked for each of the two independent variables. The results of the study shows Response Surface Counter Plot, and 3 D plot are as follows:

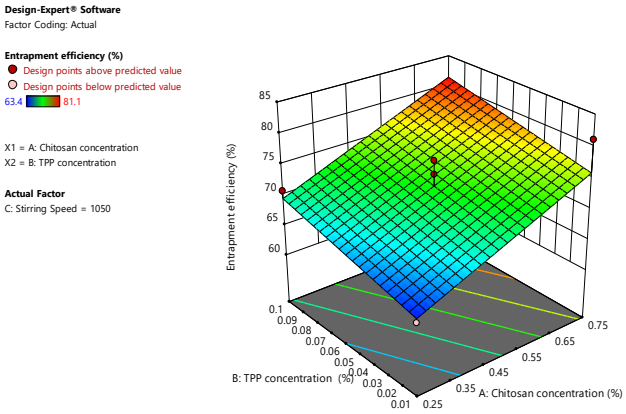

Figure 4: Effect of Chitosan and TPP concentration on entrapment efficiency shown in response surface counter plot

4.4 Particle size, zeta potential and polydispersity index

To increase the bioavailability of blueberry, it was successfully formulated as nanosuspension by a simple ionic gelation method. The particle size of nanosuspension of blueberry was 198.5nm with a PDI value of 0.351 Figure 5. The smaller the particle size, greater will be surface area and hence will improve the absorption of the drug. The optimized nanosuspension of blueberry showed higher zeta potential of 21 Mv Figure 6. Higher the value of zeta potential of a formulation, greater will be is stability[30].

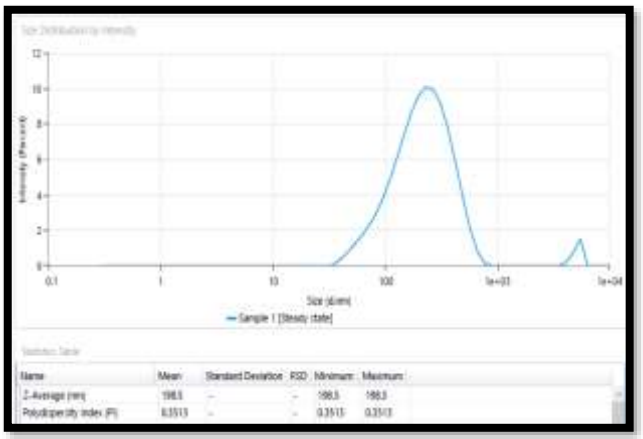

Figure 5: Particle size and PDI value of optimized formulation

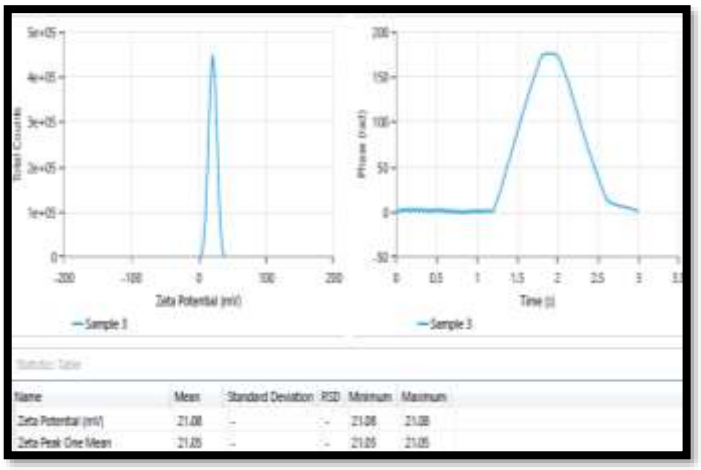

Figure 6: Zeta potential trend for blueberry nanosuspension

4.5 pH, refractive index determination

The optimized blueberry nanosuspension exhibited a pH of 6. 8±0.22 which lies in the pH range for human mucosal membrane (5-6.5) supporting the non-irritant nature of the formulation. The Refractive index was 1.33±0.02 which depicts the isotropic nature of the optimized formulation.

4.6 Percentage entrapment efficiency determination

The optimized blueberry nanosuspension exhibited the entrapment efficiency of 77.04±0.9% which is depicting better drug entrapment capability of the optimized formulation. Main challenge in formulation of nanosuspension is entrapment efficiency because the amount of drug entrapped in formulation will release to show its therapeutic effect hence more entrapment efficiency will cause more drug release and more drug release will show improved levels of drug in systemic circulation.

4.7 Assessment of surface morphology using scanning electron microscopy (SEM)

The results of scanning electron microscopy has been depicted in the Figure.7 which clearly shows that particles of optimized nanosuspension have size under 200nm which is essential characteristic of a nanoformulation. However, it also showed irregularly shaped particles with non–uniform particle size and flaky appearance.

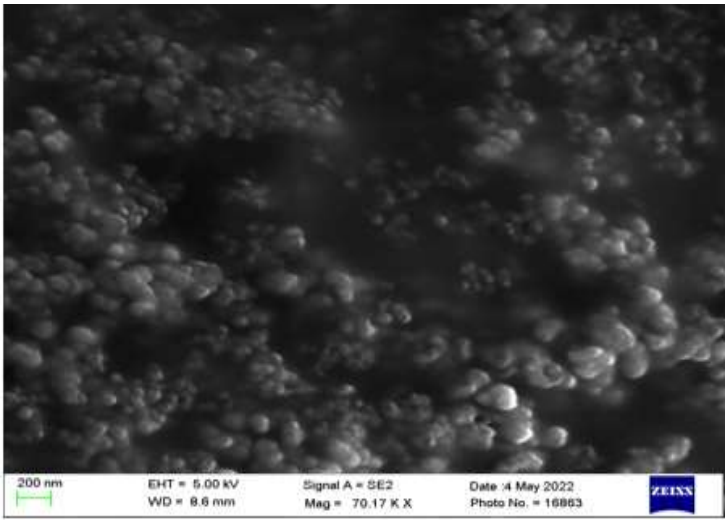

Figure 7: SEM image of blueberry nanosuspension at one resolution

4.8 Assessment of surface morphology using transmission electron microscopy (TEM)

Surface morphology was studied for optimized nanosuspension by using Morgagni 268D transmission electron microscope (FEI, Hillsbro, Holand) operated at 70 kV. The result of this surface morphology showed that particle are with in the range and spherical in shape and there is no agglomeration of particles well explained in Figure 8.

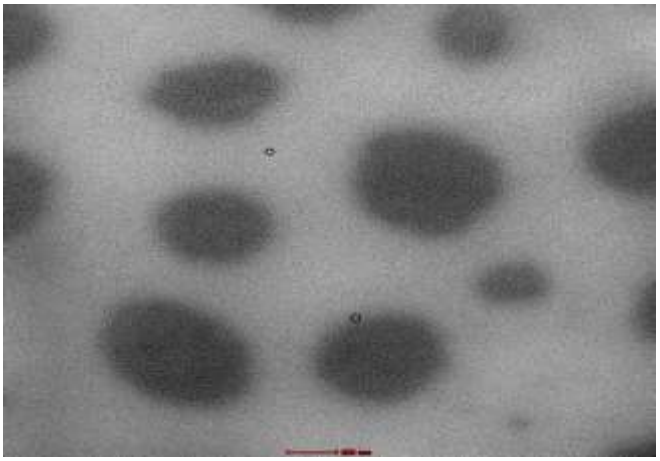

Figure.8:TEM image of blueberry nanosuspension at one resolution

4.9 Assessment of interactions between drug and excipient employing Fourier Transform Infrared Spectroscopy (FTIR)

Using a spectrophotometer (FTIR- Shimadzu Co., Kyoto, Japan), Fourier-transform infrared (FT-IR) spectra of moisture-free powdered samples of drug ,chitosan , TPP were obtained. The resolution was 1 cm^{-1}, and the scanning range was 400-4000 cm^{-1}.

Sample 1:Sodium TPP

The following distinctive bands can be seen in TPP spectra: 1210.77 cm^{-1} (P = O stretching), 1127.87 cm^{-1} (symmetric and antisymmetric stretching vibrations in PO2 group), 1086. 93 cm^{-1} (symmetric and antisymmetric stretching vibrations in PO3 group), 879 cm^{-1} (antisymmetric stretching of the P-O-P bridge)

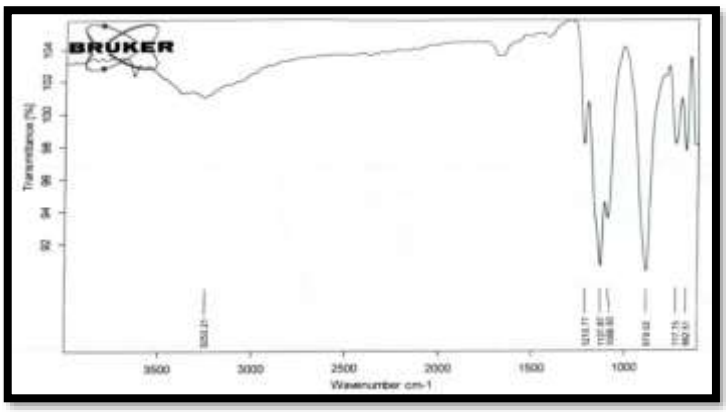

Figure 9: FTIR spectrum of Sodium Tri Polyphosphate

Sample 2: Blueberry

Among the observed characteristic peaks, the one at 3278.72 cm^{-1} represents –OH stretching vibration, the one at 2923.08 cm^{-1} represents C–H stretching vibration, the one at 1728.92 cm^{-1} represents C=O stretching vibration, the one at 1605.95 cm^{-1} represents C=C stretching vibration, and 1394.61 cm^{-1} represent C–H bending vibration, and the one at 1074.24 cm^{-1} represents C–O extension of the C–OH group.

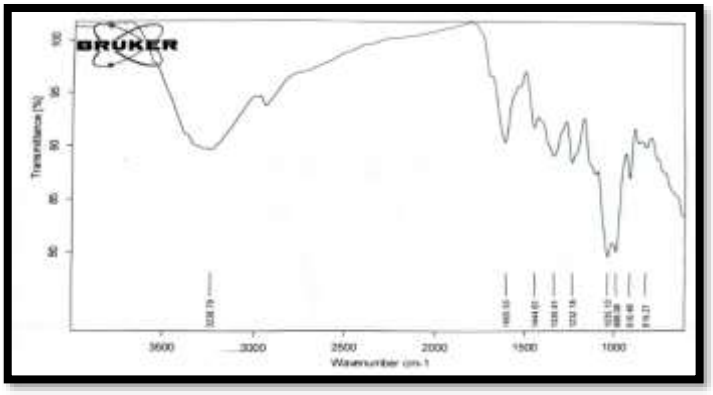

Figure 10: FTIR spectrum of blueberry

Sample 3: Chitosan

The chitosan showed a peak between 3287.7 cm^{-1} due to the presence of OH stretch band the characteristic of the hydrogen-bonded. The absorption band between 2872.37cm^{-1} showed CH stretch off CO stretching vibration conforming to aldehydes compound. The peak between 2000-2500cm^{-1} denoted the stretching vibration of *C*≡N group indicating the presence of nitriles group. The band observed between 1637. 56cm^{-1} correspond to CO stretch.

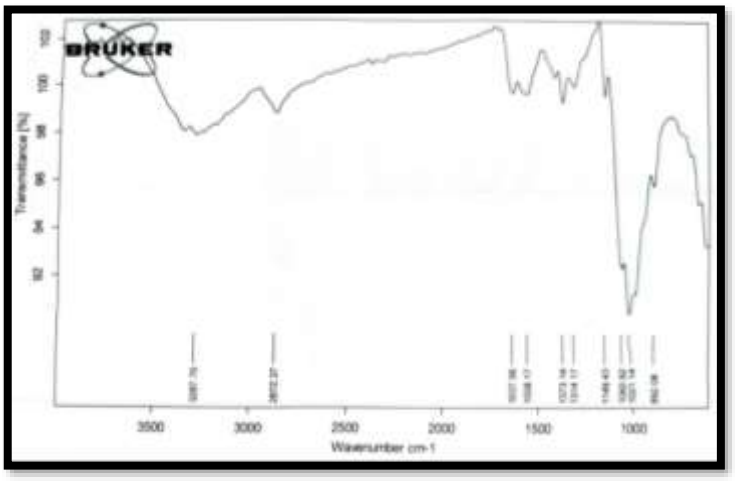

Figure 11: FTIR spectrum of chitosan

4.10 *In- vitro* drug release studies employing dialysis membrane

The % CDR from optimized blueberry nanosuspension and drug suspension was represented as a plot between % CDR and time is given in Figure 12. The optimized nanosuspension of blueberry provided biphasic drug release. In the first 20 min, it provided a fast release of 29.40% drug than the drug extract solution which provided only 23.78% drug release. After 20 minutes, the drug release from the optimized nanosuspension formulation got slow and provided a maximum drug release of 76.37% in 6 hours whereas the solution released 92.84% drug only in 2 hours which depicts the sustained release property of the optimized formulation. The optimized blueberry nanosuspension released the maximum amount of 76.3 % drug in six hours whilst drug extract solution showed the maximum drug release of 92.8% in 2 hours. The reduced drug release of optimized formulation than drug extract solution can be attributed to entrapment efficiency of the formulation because the formulation entrapped 77.04% amount of drug[31].

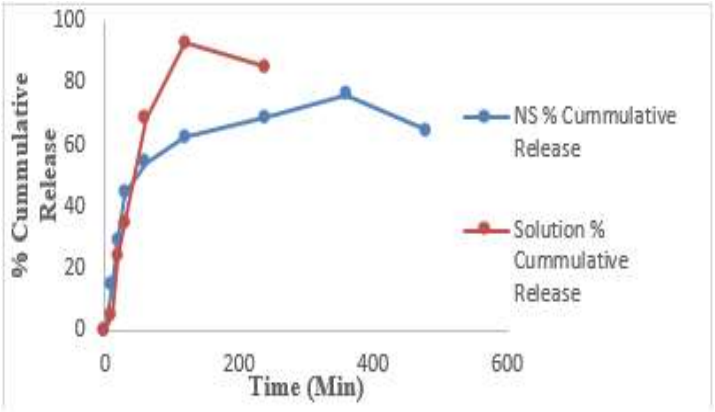

Figure 12:*In- vitro* drug release comparison of optimized blueberry formulation with coarser drug nanosuspension

4.11 Drug release model

The obtained R^2 values for various dissolution kinetic model has been summarized in Table 5. Higuchi model provided highest R^2 value of 0.879 among all dissolution kinetic models which suggested that the drug release from optimized drug formulation follows higuchi model which has been depicted in the Figure 13.

Table 5: Comparision of different kinetic model

S. NO	MODEL	R^2 VALUE
1	Zero order	0. 650
2	First order	0. 779
3	Higuchi	0. 879
4	Hixson crowell	0. 736

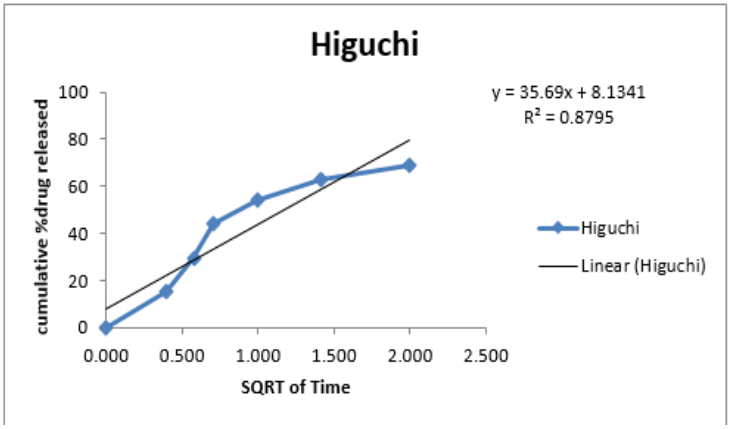

Figure 13: Higuchi model

5. STABILITY STUDIES

The main issue with nanosuspension formulation is to maintain stability through its shelf life. The particle size of prepared nanosuspension at room temperature of (25℃-30℃) was 198.5nm with a PDI value of 0.351. The physical appearance of this nanosuspension was clear and stable. The particle size of prepared nanosuspension at refrigerated conditions of (2-8℃) was 197.5 and the PDI value 0.341.The physical appearance of the nanosuspension is stable. Result of stability testing of optimized Blueberry nanosuspension revealed in Table 6. that nanosuspension remains stable in refrigerated conditions. On the other hand, the nanosuspension present at room temperature shows clear and stable physical. The insignificant decrease in the particle size (197.5with PDI value of 0.341) of nanosuspension was seen under refrigerated conditions (2-8℃). After a periodic interval of time, the sample was withdrawn and analyzed for each storage condition. The data generated after storage stability studies for optimization of blueberry nanosuspension has been summarized in given Table 6.

Table 6 : Stability studies of optimized nanosuspension

	Parameters	**After 1 month**	**After2 months**	**After3 month**
Freshly prepared nanosuspension	Size	198. 5	198.1	199.2
	PDI	0. 351	0.341	0.331
	Physical appearance	Clear and stable	Clear and stable	Clear and stable
	Entrapment efficiency	72.01%	73%	75%
Room temperature (25℃ -30℃	Size	199. 5	195.4	198.2
	PDI	0. 331	0.351	0.341
	Physical appearance	Clear and stable	Clear and stable	Clear and stable
	Entrapment efficiency	74%	75.01%	76%
Refrigerated condition (2-8℃)	Size	197. 5	199.1	197.4
	PDI	0. 341	0.321	0.345
	Physical appearance	Clear and stable	Clear and stable	Clear and stable
	Entrapment efficiency	72.04%	74.03%	74.01%

CONCLUSION:

Blueberry nanosuspension was successfully prepared by the ionic gelation techninque. The Box Behnken design was applied for optimization of the formulation with three independent variables chitosan concentration, TPP concentration ,and stirring speed along with two dependent variables particle size and entrapment efficiency. The result of following parameters are particle size of 205.05 nm and entrapment efficiency of 76.60 % will be an optimized formulation if it has chitosan, TPP, and stirring speed of 0.5%, 0.05%, and 1050 rpm respectively. In addition, it was found to have polydispersity index , refractive index, zeta potential of 0. 351,1.33, 21.08mv respectively.SEM analysis showed the droplet size under 200nm. Dissolution study revealed the biphasic release of drug from nanosuspension which was 29% in first 20 min and maximum release was obtained after 6 hours whereas the solution of plant extract release of drug is 92.8% only in 2 hours. After all observations, it was determined that nanosuspension is an effective method for delivering drugs for enhancing the poor systemic bioavailability of blueberry by maintaining the optimum concentration because if the concentration is higher or lower then it will not show beneficial effects, and by comparing drug extract suspension with nanosuspension it was found that the drug's release of extract is within 2 hr about 92.8% which shows the immediate release of drug which is not health beneficial because as per literature animal studies indicate that high dose of polyphenol supplements may damage the kidney, causes tumors or unbalance thyroid hormone, gastric irritation due presence of salicylate so to overcome this nanosuspension is a promising technique for health benefits.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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