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Formulation, Optimization and Evaluation of Nanoparticulate In-Situ Gel of Anthocyanin from Bilberry for Ocular Inflammation

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ABSTRACT

A natural source of anthocyanins that is highly concentrated is the bilberry (Vaccinium myrtillus L.). Bilberries and other berry fruits are known for their numerous health advantages. These polyphenolic components are thought to be the primary bioactive responsible for the bilberry's blue/black hue and strong antioxidant content. Even yet, the main benefit of bilberry is often touted as improved eyesight. Most people are most familiar with bilberries because of their supposed benefits for eyesight, especially at night. According to 2-year research, consuming 120 mg of bilberry anthocyanins daily improved visual performance by around 30% in persons with glaucoma, a disorder that causes progressive blindness; in contrast, the placebo group had a worsening visual function. Ionotropic gelation was used to create nine distinct batches (F1–F9) of bilberry-loaded Nanoparticulate In-situ gel, which was then homogenized to minimize size. When encapsulation efficiency (EE%) of the resultant Nanoparticles was measured, it was found that formulation F3 (2 gm calcium chloride, 3 gm sodium alginate) had the greatest EE% of Bilberry loaded Nanoparticles, 92.33±0.38. An assessment of the glaucoma efficacy was conducted using a selected batch (F3) combined with chitosan, HPMC, and Pluronic F127 gel. The drug release of formulation F1-F3 was found to be 82.11, 88.12, 93.0 % respectively. The drug release formulation F3 being the maximum.

Keywords: *Nanoparticulate, in-situ gel, anthocyanin, bilberry, ocular, inflammation*

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1. Introduction

1. Introduction

Ocular inflammation is a broad term that refers to a variety of eye conditions that can cause discomfort, redness, and visual impairment, such as keratitis, conjunctivitis, and uveitis. Infections, inflammatory reactions, trauma, and irritation exposure are frequently the root causes of these disorders. Ocular inflammation can lead to serious consequences, including cataracts, glaucoma, and even irreversible vision loss, if treatment is not received. Nonsteroidal anti-inflammatory medications (NSAIDs) and corticosteroids are used as part of the current standard therapy protocol. Although these treatments work well, they have serious adverse effects that include elevated intraocular pressure, cataract development, and systemic consequences from continuous usage. Because of this, there is an urgent demand for safe and efficient alternative medicines.[1-4]

Naturally occurring pigments called anthocyanins, a subclass of flavonoids, are present in many fruits and vegetables, most notably bilberries (*Vaccinium myrtillus*) and other berries. These substances are well known for their anti-inflammatory, antioxidant, and vasoprotective qualities. The anthocyanins found in bilberries have long been utilized in herbal medicine to promote eye health and enhance vision. By showing that anthocyanins can prevent the synthesis of pro-inflammatory mediators, shield retinal cells from oxidative stress, and improve ocular microcirculation, contemporary research has confirmed these traditional applications. Anthocyanins are a viable option for the management of ocular inflammation because of these characteristics.[5-7]

Although anthocyanins have potential advantages, their quick metabolism in the body, low bioavailability, and poor stability restrict their therapeutic applicability. New developments in medication delivery technologies have been investigated in an effort to address these issues. The creation of nanoparticulate delivery systems that can improve the solubility, stability, and

bioavailability of bioactive chemicals is made possible by nanotechnology, which in particular provides a viable approach. Nanoparticles can allow targeted transport to certain regions, offer regulated release, and shield delicate substances from destruction.[8-10]

The application of nanoparticulate systems provides further benefits in the context of ocular medication delivery. Effective medication distribution is hampered by the corneal barrier, tear dilution, and fast drainage through the nasolacrimal duct, among other peculiarities of the eye's architecture and physiology. These issues can be resolved by longer retention times on the ocular surface, improved penetration across ocular barriers, and less frequent delivery of nanoparticulate systems. A technique that shows promise is the creation of in-situ gels, which are liquid formulations that change into gels when they come into contact with the physiological conditions of the eye, such as pH or temperature. By lengthening the medication's duration of residency on the ocular surface, this change improves drug absorption and therapeutic effectiveness.[11-13]

In conclusion, the integration of nanotechnology with natural bioactive compounds like anthocyanins offers a promising avenue for the development of novel ocular therapeutics. The formulation of a nanoparticulate in-situ gel containing bilberry anthocyanins could provide a safe and effective alternative for the treatment of ocular inflammation, potentially improving patient outcomes and reducing the burden of eye diseases.

2. Materials and Methods

2.1 Materials

Drug sample and chemical reagents used in the formulation of liposomal gel of Atorvastatin were procured from different reputed companies.

2.2 Experimental work

2.2.1 Determination of λmax

A Shimadzu UV-1800 spectrophotometer was used to measure the bilberries' UV range. A final volume of 10 milliliters was obtained by breaking exactly 10 milliliters of bilberry in a sufficient amount of distilled water. A 0.25 ml aliquot from the stock solution was withdrawn, and the volume was increased to 25 ml with distilled water to achieve a centralization of 10 µg/ml after the stock arrangement had been attenuated to acquire a convergence of 1000 µg/ml. After testing the final configuration between 200 and 800 nm, the greatest value was identified by recording the range.

2.2.2 Preparation of standard curve of bilberry in distilled water

The bilberry standard curve was first prepared by combining 10 mg of bilberry with 100 ml of

distilled water in a volumetric flask. The medication was broken down in the distilled water using an ultra-sonication. Four distinct dilutions of the stock solution (0.1 mg/ml) were made, each with a concentration of 0.05 mg/ml, 0.025 mg/ml, 0.0125 mg/ml, and 0.0062 mg/ml. A UV-Visible Spectrophotometer (Shimadzu, Japan) was then utilized to analyze the produced dilutions at 517 nm.

2.2.3 Determination of anthocyanins

The sample's total anthocyanins content was calculated using an UV-Vis spectroscopy.

2.2.3.1 Preparation of buffers

To prepare the pH 1.0 buffer (0.025 M potassium chloride), weigh 0.93 g of potassium chloride in a 500 ml beaker using an analytical balance. Next, add 490 ml of pure water. A pH meter was used to determine the pH, and HCl was used to get the pH up to 1.0. After the solution was moved to a 500 ml volumetric flask, distilled water was added to raise the volume to 1000 ml. To prepare the pH 4.5 buffer (0.4 M sodium acetate), weigh 27.21 g of sodium acetate using an analytical balance and add 490 ml of distilled water to the 500 ml beaker. A pH meter was used to measure the pH, and HCl was used to bring it down to pH 4.5. After the solution was moved to a 500 ml volumetric flask, distilled water was added to raise the volume to 1000 ml.

2.2.3.2 Preparation of test solutions

A bilberry stock solution with a concentration of 1 mg/ml. The test bilberry was diluted with a pH 1.0 buffer until the spectrophotometer absorbance at 517 nm was within the linear range, at which time the dilution factor was computed. The test component was delivered in a quantity of no more than 10 ml. The estimated dilution factor 2 test solutions for each fruit were created, the first with a pH 1.0 buffer and the second with a pH 4.5 buffer.[14]

Calculation: The anthocyanin pigment concentration was calculated using the following equation.

$$\text{Anthocyanins pigment} = \frac{A.MW.DF.10^3}{E.L}$$

Where:

$A = (A_{520nm}-A_{700nm})\ pH_{1.0} - (A_{520nm}-A_{700nm})\ pH_{4.5}$

MW = 449.2 g MOL^{-1} for $cyaniding^{-3}$ glucoside

DF = Dilution factor

L = Path length

E = 26,900 molar extinction coefficient, in L/Mol cm for cyanidine-3-glucoside

10^3 = Factor for conversion from g to mg

2.2.3.3 Drug excipients compatibility study

2.2.3.3.1 Fourier transforms infrared spectroscopy (FTIR)

FTIR was used to investigate drug compatibility for sodium alginate, the physical combination, and the pure drug. The results demonstrated that bilberry and sodium alginate, both of which are used to create nanoparticles, are not chemically incompatible.[15]

2.2.4 Formulation, Development & Evaluation

2.2.4.1 Preparation of bilberry loaded Nanoparticles by ionotropic gelation techniques

The process of creating bilberry-loaded sodium alginate nanoparticles involved modifying the ionotropic gelation procedure. Different amounts of sodium alginate were dissolved in distilled water using a magnetic stirrer, and the mixture was then allowed to stand for thirty minutes. The medication (bilberry) was then stirred while suspended in the sodium alginate solution previously described. A suspension of bilberry, sodium alginate, and calcium chloride nanoparticles was created by gradually adding different amounts of calcium chloride solution to the drug and polymer solution that had previously been created. In order to encourage extended drug release, calcium chloride was employed as a cross-linking agent for the sodium alginate nanoparticles. It was sonicated for twenty-five minutes. Following sonication, the suspension of nanoparticles was centrifuged at 10,000 rpm for 30 minutes (Remi CPR-30 PLUS), and the supernatant was extracted. After being collected, the pellet was once again dispersed in demonized water, sonified, centrifuged, and lyophilized.[16]

Table 1. Experimental design of sodium alginate nanoparticles of bilberry drug

Batch No.	Drug (mg)	Calcium Chlorid (gm)	Sodium Alginat (gm)
1	100	1	2
2.	100	1.5	2.5
3.	100	2	3
4.	100	2	2
5.	100	1	2.5
6.	100	1	3
7.	100	1.5	2
8.	100	2	2.5

9.	100	1.5	3

2.2.5 Evaluation of nanoparticles

2.2.5.1 Particle size

Zeta potential (ζ) was used to characterize nanoparticles using a Zetasizer 4 (Malvern Instruments Ltd., Malvern, UK).

2.2.5.2 Zeta potential (ζ)

The zeta potential (ζ) of nanoparticles was measured with a zetasizer 4 (Malvern Instruments Ltd., Malvern, UK). Using an automated mode, an aqueous dip cell measured the zeta potential. Samples were put in a capillary measuring cell with the cell location changed after being diluted in ultrapurified water.

2.2.5.3 Determination of encapsulation efficiency (EE) of nanoparticles

Remi's CPR-30 PLUS centrifuged a 10 ml solution of nanoparticles for 90 minutes at 10 °C and 10,000 rpm. The clear supernatant that was left over after centrifugation was diluted ten times with double-distilled water, and the quantity of bilberry was quantified with a UV-Visible spectrophotometer that had been calibrated to have a maximum wavelength of 517 nm. Efficiency of Encapsulation.[17]

$$\text{Encapsulation efficiency} = \frac{\text{Total amount of bilberry} - \text{Amount of free bilberry}}{\text{Total amount of Bilberry}} \times 100$$

2.2.5.4 Percentage yield

Nanoparticles were gathered and precisely weighed. The following procedure was then used to compute the percentage (percent) yield[18]:

$$\% \text{ Yield} = \frac{\text{Mass of nanoparticles obtained}}{\text{Total weight of drug and polymer}} \times 100$$

2.2.5.5 In-vitro drug release of bilberry

All nine batches of bilberry nanoparticles were discharged using the dialysis bag method (Batch 1 through Batch 9). Drug-loaded nanoparticles weighing 30 mg were added to a USP dissolving test apparatus that has a basket-style stirring device. Within the basket was the dialysis bag. The dissolving media, a phosphate buffer solution with a pH of 7.4, was maintained at 37°C. The speed at which the basket was revolving was 100 rpm. At intervals of 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0 hours, 5.0ml of the medium were removed using a 5.0ml syringe and replaced with

5.0ml of phosphate buffer solution (pH 7.4). To find the drug content, a UV spectrophotometer with a 517 nm setting was used.[19] The formulation with highest EE% and drug release were selected for further studies.

2.2.5.6 Microscopic evaluation

Using an optical microscope at 10x magnification, the produced Nanoparticle were examined for morphological structure, visual size estimation etc.

2.2.5.7 Scanning Electron Microscope (SEM)

A SEM was used to analyze the particle size of the formulation.

2.2.6 Formulation of bilberry loaded nanoparticulate in-situ gel

2.2.6.1 Preparation of nanoparticulate *in situ* gel formulations

The necessary amount of chitosan was dissolved in a solution of acetic acid (2% w/v) while stirring continuously until the chitosan was entirely dissolved, to create the chitosan solutions (0.1–0.3% w/w). The required quantity of polymer was dispersed in distilled, deionized water and continuously stirred for one hour at room temperature to create Pluronic solutions (1.5–2.5% w/w) and HPMC 0.5gm. Only partially dissolved pluronic solutions were kept in the refrigerator (4°C) for around 24 hours, or until the entire polymer was dissolved. The needed amount of Pluronic was dissolved in the desired concentration of chitosan with constant stirring for an hour to create the chitosan/Pluronic, HPMC solutions. After that, the partially dissolved solutions were cooled down until the combination was finished, which took around 24 hours. The final concentrations of chitosan and pluronic, together with the HPMC component of the mixture, were included in the composition of the chitosan/Pluronic mixture that was specified. The chitosan/Pluronic, HPMC solutions were continuously spun while 0.3 percent of the Nanoparticle was added to create bilberry-loaded Nanoparticle-containing polymer solutions. A Benzalkonium chloride solution containing 0.003 percent preservative was added to each solution. Every sample solution had its pH adjusted to either 7.4 or 4.0 ± 0.1.

2.2.6.2 Physical parameters

The formulated in situ gel solution was tested for clarity, pH, gelling capacity, and drug content estimation.

2.2.6.2.1 Gelling capacity

The formulation's ability to gel was assessed by placing a drop of the produced formulation into a vial holding two milliliters of recently made artificial tear fluid and observing the results. The duration of the gelling process was noted.[20-23]

2.2.6.2.2 Drug content estimation

By diluting the created formulation in 100ml of distilled water and measuring it at 517 nanometers with a UV-visible spectrophotometer (Shimadzu UV-1700 PC, Shimadzu Corporation, Japan), the drug concentration was determined.

2.2.6.2.3 In vitro permeation studies

1gm of Nanoparticulate-loaded in situ gel solution was placed into a dialysis bag and sealed with a rubber band after spending the night in artificial tear fluid with a pH of 7.4. This was done to look at the in vitro diffusion of gel across a membrane. The above-mentioned cellophane membrane was gelled before being dissolved in an identically-temperatured liquid containing artificial tear fluid. At 340 degrees Celsius and 50 rpm, the stirring was carried out. At regular intervals, 5 mL aliquots of the release medium were taken out and replaced with an equivalent volume of fresh medium for the sample. Using a UV spectrophotometer set at 517 nm, the amount of medication released after aliquots were diluted with simulated tear fluid was determined.[24,25]

3. Results and Discussion

3.1 Extract characterization

3.1.1 Determination of λmax

The λ_{max} of 10µg/ml solution of bilberry solution in distilled water was found to be 517nm. Figure 1. shows λ_{max} of bilberry in distilled water

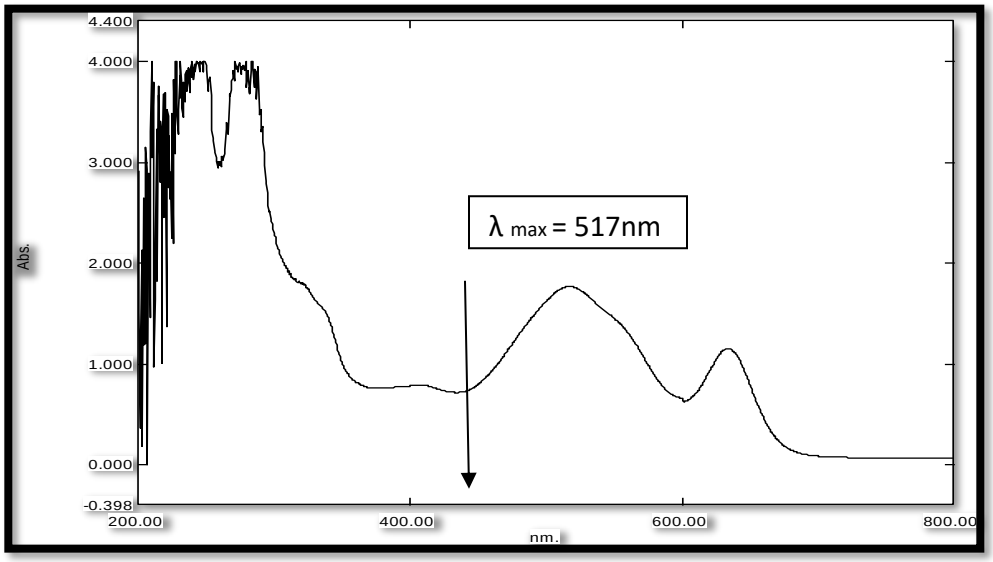

Figure 1. λ_{max} of bilberry in distilled water

3.1.2 Calibration curve of bilberry

The UV absorption information at 517 nm wavelength is displayed in Figure 2 and Table 2. Bilberry was shown to have high linearity in the 2–10 µg/ml range ($y = 0.0008x + 0.0003$ and $r^2 = 0.9995$).

Table 2. Absorbance of bilberry

S. No.	Conc.	Abs.
1	0	0
2	2	0.009
3	4	0.018
4	6	0.026
5	8	0.032
6	10	0.044

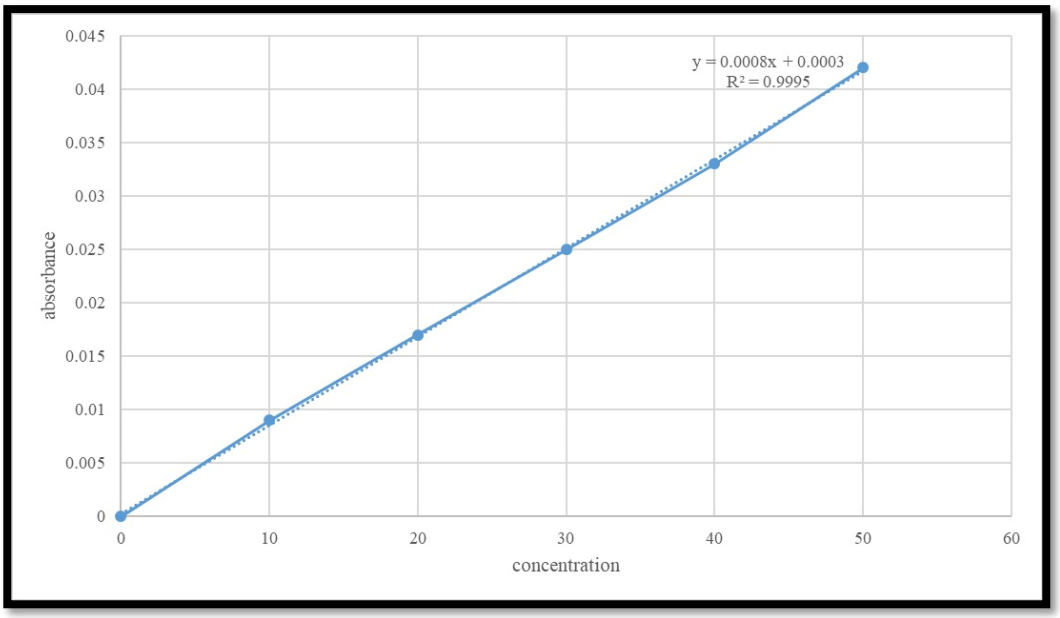

Figure 2. Calibration curve of bilberry

3.2 Compatibility Studies

3.2.1 FTIR Spectroscopy

By comparing the extract's spectrum with that of the individual excipients and extract with that of the herbal extract-excipients complex, FTIR spectroscopy assists in verifying the creation of the complex between the excipients and extract.

3.2.1.1 FTIR of bilberry

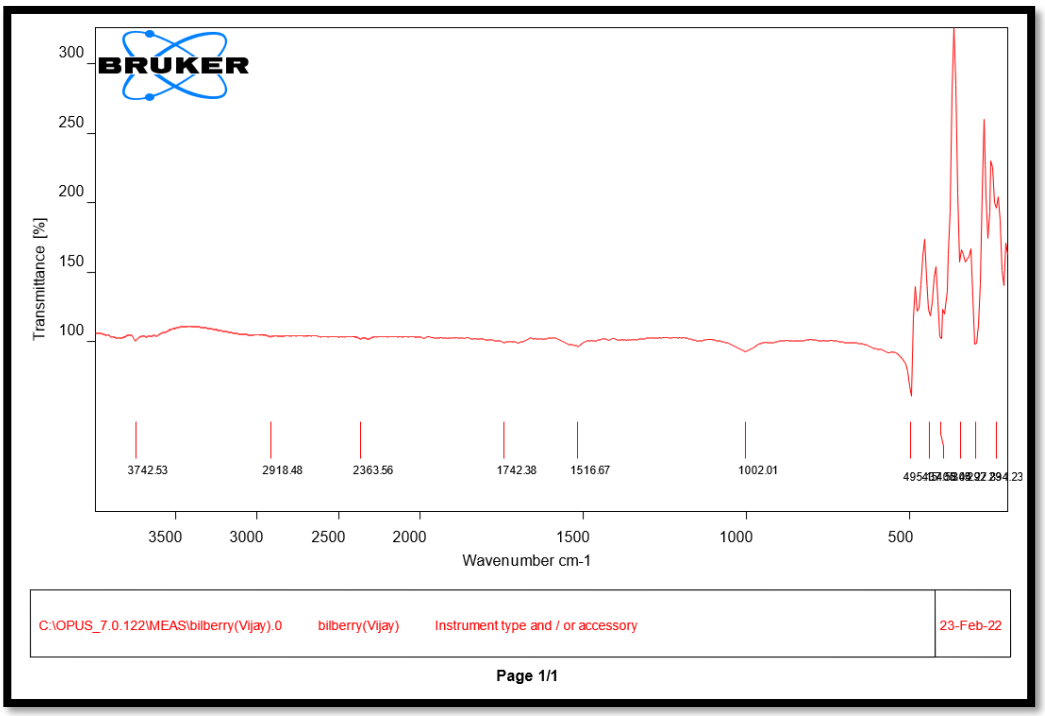

Figure 3. FTIR of bilberry

3.2.1.2 FT-IR of physical mixture of bilberry and sodium alginate

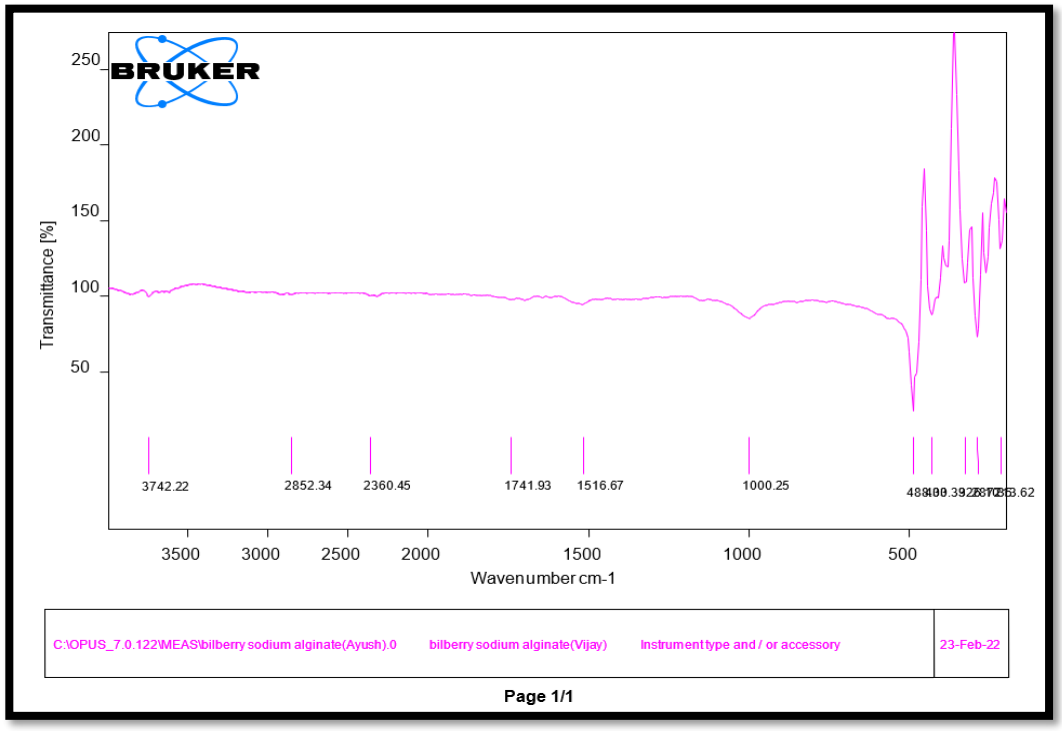

Figure 4. FT-IR of physical mixture of bilberry and sodium alginate

3.2.1.3 FT-IR of physical mixture of bilberry and sodium alginate and calcium chloride

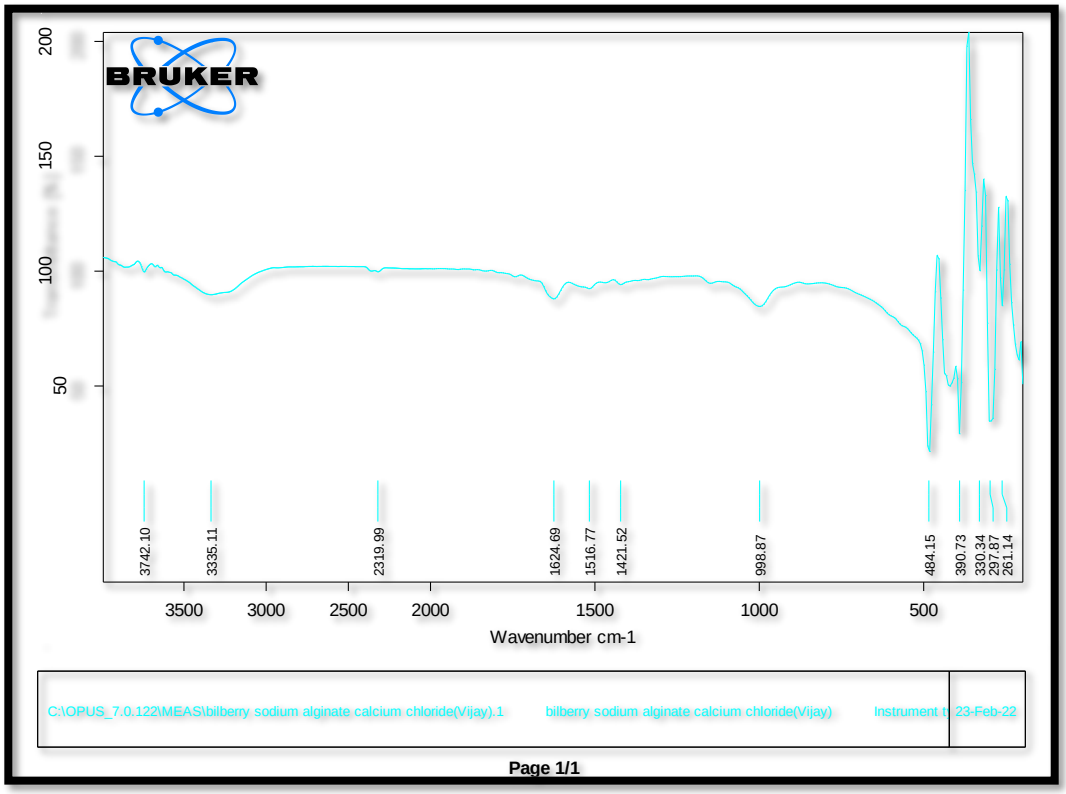

Figure 5. FTIR of physical mixture of bilberry and sodium alginate and calcium chloride

3.3 Evaluation of Nanoparticles

3.3.1 Particle size

Particle size and size distribution are the most significant features of the nanoparticles system; the sizes of the bilberry-loaded nanoparticles varied from 707.7 nm to 999.7 nm, indicating that the particles decrease with increase in polymer concentration. The sizes of the nanoparticles were measured using zeta potential (ζ) using a Zetasizer 4 (Malvern Instruments Ltd., Malvern, UK).

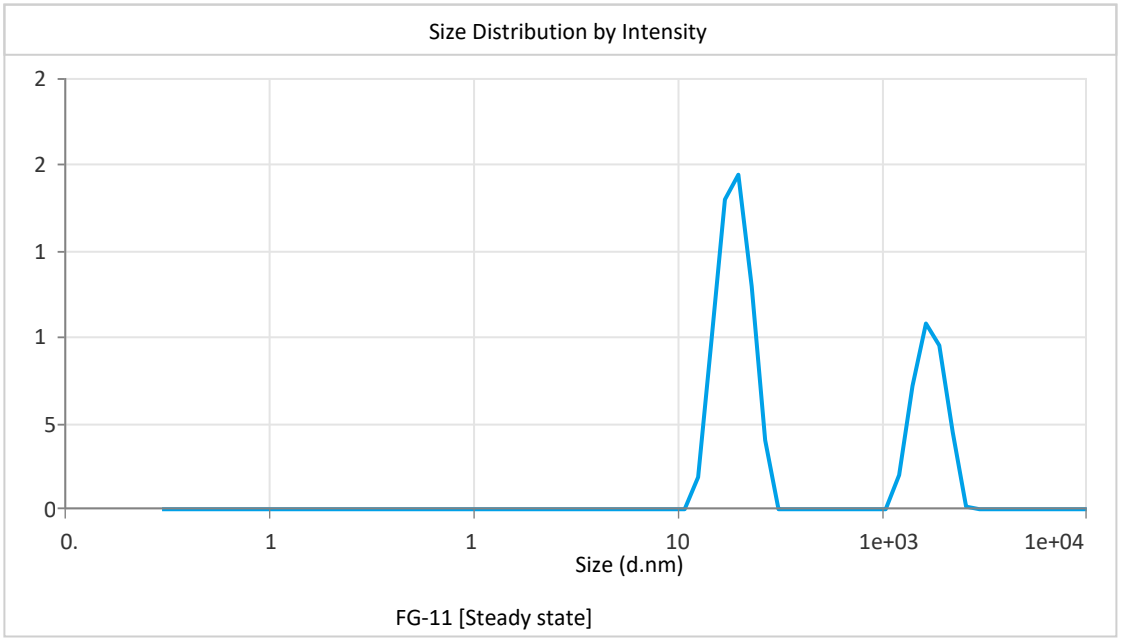

Figure 6. Zeta sizer of F3 Batch of Nanoparticle and particle size was found to be 707.7nm

3.3.2 Zeta potential

Zeta potential of drug loaded NPs were found to be −31mV and −41mV, respectively (Table 3).

Table 3. Zeta potential of drug loaded NPs

Formulation	Zeta Potential (mV)
F1	-34
F2	-33
F3	-31
F4	-34
F5	-41
F6	-40
F7	-36
F8	-36
F9	-35

3.3.3 Encapsulation Efficiency

Then the amount of active constituent in the supernatant was determined using UV spectrophotometer at 517 nm and the absorbance readings were used to calculate the amount of free bilberry-drug which further determines the EE%. The EE% of all the batches were calculated which is mentioned in table 4 and figure 7. The EE% ranged between 72.7 to 92.33%.

Table 4. EE% of different batches of formulated bilberry loaded nanoparticles

Batch	Encapsulation Efficiency (EE%)
F1	82.6±0.26
F2	87.56±0.31
F3	92.33±0.38
F4	82.56±0.20
F5	85.33±0.35
F6	88.75±0.20
F7	85.26±0.37
F8	72.7±0.30
F9	81.5±0.40

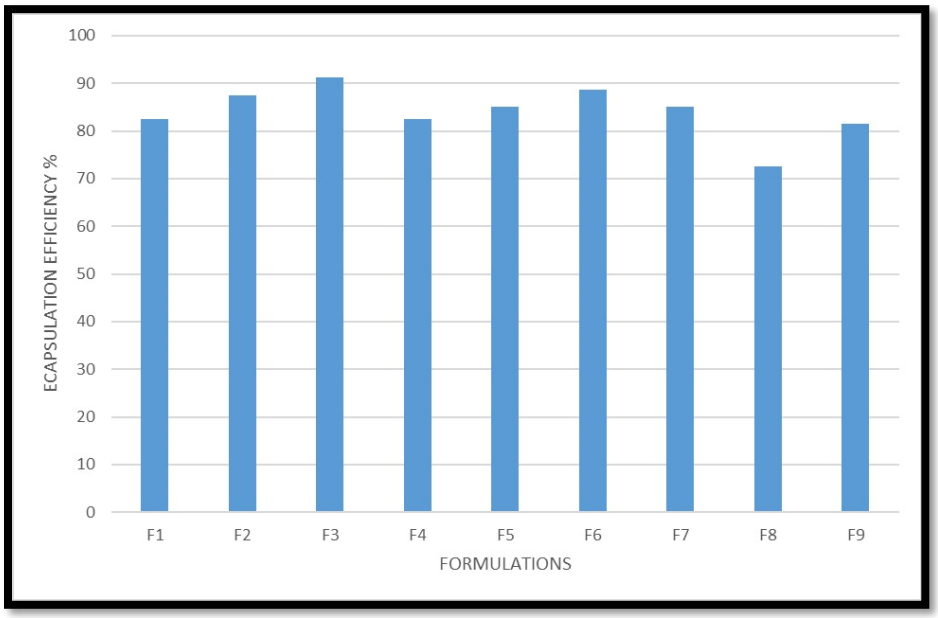

Figure 7. EE% of different Nanoparticle batches.

3.3.4 Percentage yield in nanoparticles

The amount of bilberry loaded Nanoparticles in each sample was determined by formula. The percentage yield of formulations F1-F9 was found 61.93, 61.46, 79.21, 75.12, 70, 59.51, 55, 64.56 and 67.82 respectively. The formulation F3 shows the maximum drug content. The percentage yield for all batches of Nanoparticle depicted in Fig 8, table 5.

Table 5. Percentage yield of nanoparticles

Batches of Nanoparticle	Percentage yield
F1	61.93±0.11
F2	61.46±0.28
F3	79.21±0.30
F4	75.12±0.29
F5	70.±0.49
F6	59.51±0.34
F7	55.±0.48
F8	64.56±0.43
F9	67.82±0.23

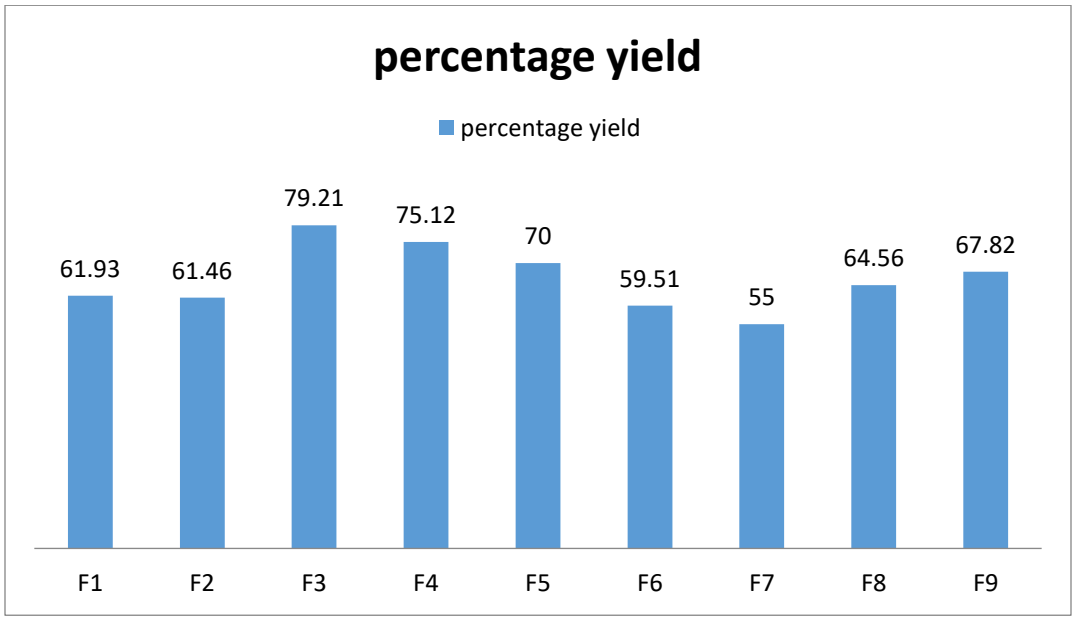

Figure 8. Percentage yield of Nanoparticles

3.3.5 In-vitro drug release of bilberry nanoparticles

The in vitro drug release studies of bilberry loaded Nanoparticle ranged from 78.84% to 92.96%, with the formulation F3 exhibiting the highest in-vitro drug release of 92.96% at 6 hours. The dissolution profile of all the batches of bilberry loaded Nanoparticle was obtained in phosphate buffer at pH 7.4. The in vitro dissolution testing was conducted for 12 hours.

Table 6. *In-vitro* drug release of bilberry loaded nanoparticles F1-F9

Time (hr)	F1	F2	F3	F4	F5	F6	F7	F 8	F9
1	3.75±	3.75±	5.29±0	3.43±0	4.12±0	3.76±0	3.78±0	4.25±0	4.22±0
2	10.51±0	10.73±0	13.23±0	10.52±0	10.16±0	10.86±0	10.63±0	10.43±0	9.73±0
4	19.53±0	19.34±O	22.54±0	19.24±0	17.56±0	19.44±0	18.2±0	17.71±0	17.80±0
6	30.72±0	30.71±0	32.3±0	28.88±0	27.36±0	29.65±0	27.8±0	26.71±0	28.52±0
8	46.79±0	47.45±0	49.22±0	43.88±0	42.67±	44.38±0	41.71±0	39.31±0	41.57.0

10	66.45±0	67.52±0	69.34±0	61.23±0	61.20±0	61.59±0	59.38±0	55.76±0	58.76±0
12	88.24±0	90.45±0	92.96±0	83.32±0	82.45±	82.27±0	81.78±0	78.84±0	83.30±0

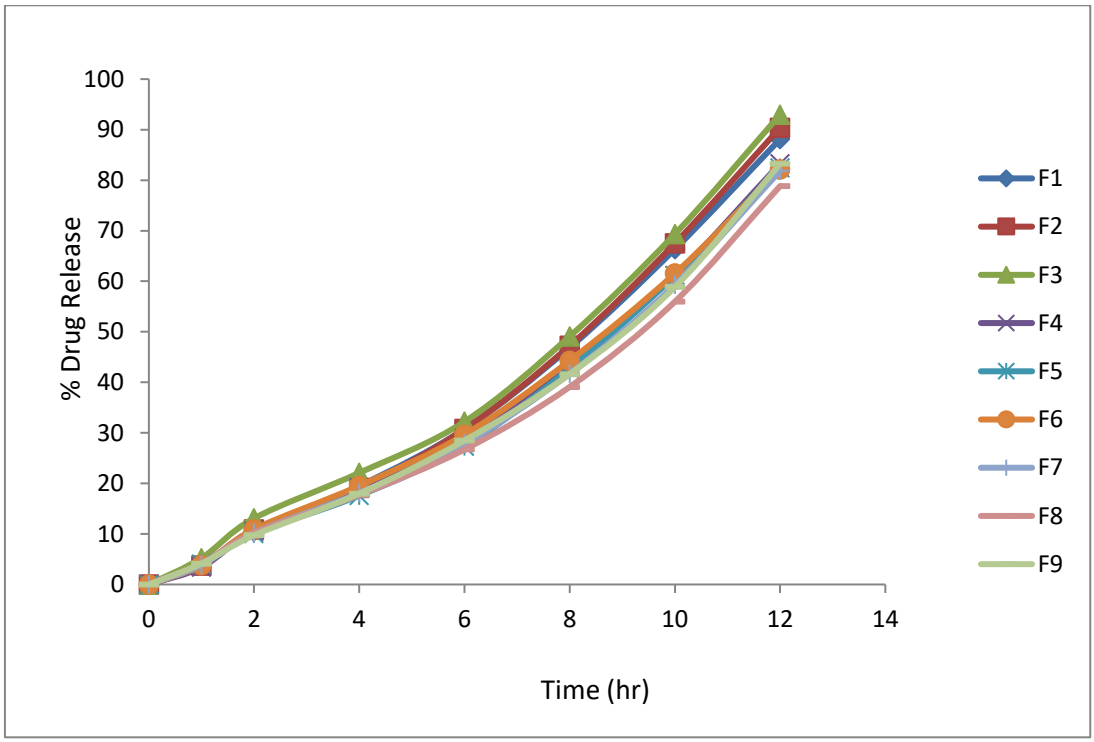

Figure 9. % Drug Release of bilberry loaded nanoparticles (F1-F9)

Based on the result obtained from EE%, Percentage yield and *in-vitro* drug release profile, the batch F3 showed better results when compared with other batches.

3.3.6 Microscopic evaluation

The prepared bilberry loaded nanoparticles under microscope has shown characteristic features of presence of projection round/spherical shape vesicles Figure 10 at the magnification of 10x.

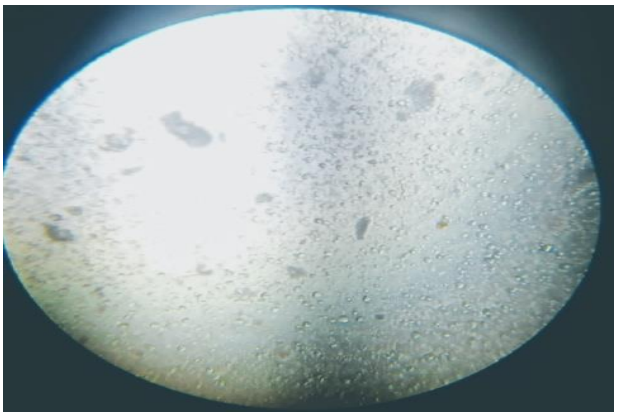

Figure 10. Microscopic view of bilberry loaded nanoparticles formulation (F3) 10x.

3.3.7 SEM

The SEM images were seen for the nanoparticles formulations. The results are shown in Figure 11.

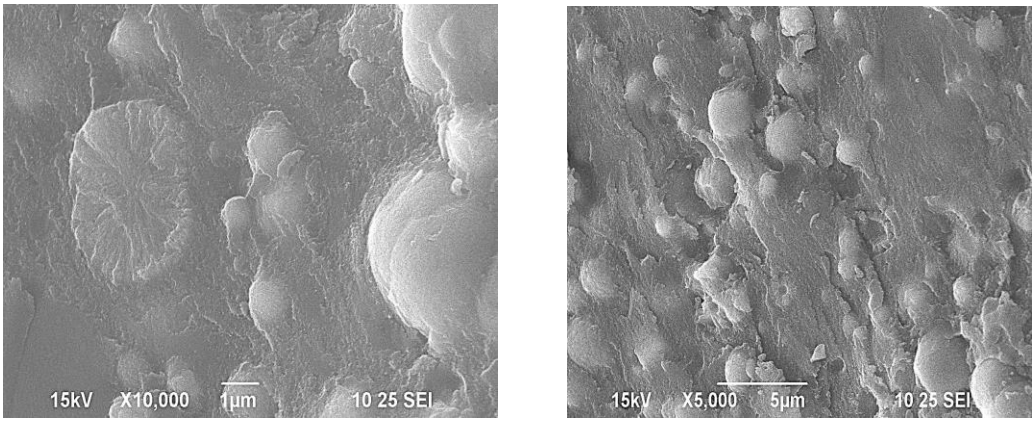

Figure 11. SEM image of bilberry loaded nanoparticles formulation (F3)

3.4 Evaluation of bilberry loaded nanoparticulate in-situ gel

3.4.1 Physical parameters

The formulated in situ gel solution was tested for clarity, pH, gelling capacity, and drug content estimation. The results are as shown in Table 7.

3.4.2 pH

The pH was measured of bilberry loaded nanoparticulate in-situ gel using Decibel digital pH meter. The pH of formulation F1-F3 was found 6.62, 6.95, and 7.0 respectively. All measurement of pH is given in Figure 12 and table 7.

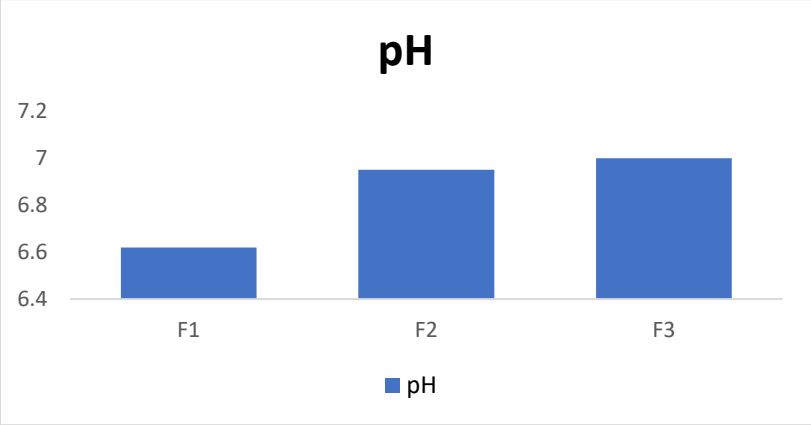

Figure 12. pH of formulation F1-F3

3.4.3 Drug content

The amount of bilberry loaded nanoparticulate in-situ gel in each sample was determined by spectrophotometer (UV-1800, Shimadzu). The UV absorbance of the sample was determined at a wavelength of 517nm. The drug content of formulations F1-F3 was found 87.6, 89.2, and 91.3% respectively. The formulation F3 shows the maximum drug content. The drug content for all batches of in-situ gel is depicted in Figure 13, Table 7.

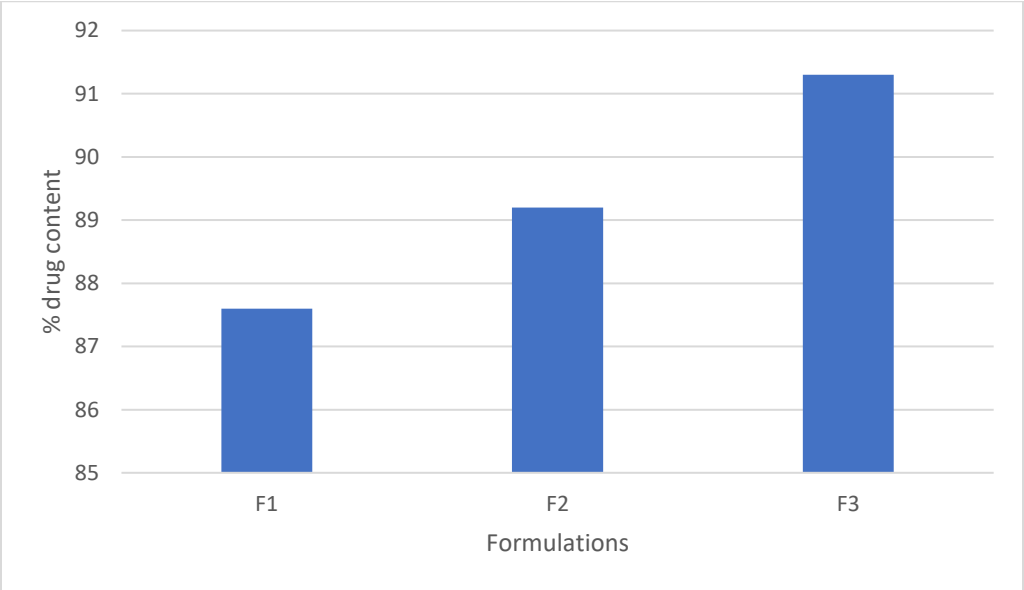

Figure 13. % Drug content of formulation F1-F3

Table 7. Evaluation data for all F1-F3 batches of in-situ gel

Formulations	Clarity	Visual appearance	pH	Drug conte	Gelling capacity
F1	Clear	Transparent	6.62	87.6	++
F2	Clear	Transparent	6.95	89.2	++
F3	Clear	Transparent	7.0	91.3	++

3.4.4 *In vitro* drug release

The amount of drug release is an important parameter for controlled release formulation. The drug release of formulation F1-F3 was found to be 82.11, 88.12, 93.0 % respectively. The drug release formulation F3 being the maximum. The drug release data of all the formulation is depicted in Figure 14 and Table 8 shows a release.

Table 8. *In-vitro* drug release of bilberry loaded nanoparticulate in-situ gel F1-F3

Time (hr)	F1	F2	F3
0	0	0	0

1	3.36	3.74	3.75
2	8.99	10.12	10.87
4	16.86	19.12	19.87
6	27.74	29.99	31.50
8	43.86	46.49	47.62
10	62.61	65.99	67.12
12	82.11	88.12	93.00

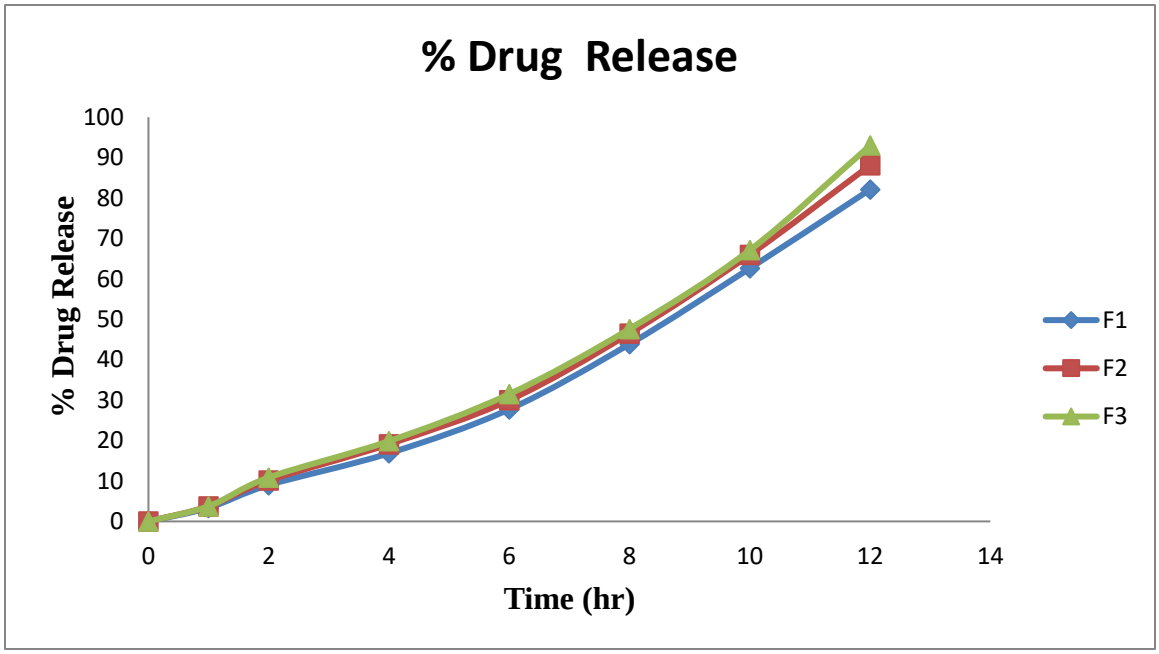

Figure 14. %Drug Release of bilberry loaded nanoparticulate in-situ gel F1-F3

4. Conclusion

The bilberry (Vaccinium myrtillus L.) is a highly concentrated natural source of anthocyanins. Berries, including bilberries, are well-known for their many health benefits. These polyphenolic components are believed to be the main bioactive that gives bilberries their significant antioxidant capacity and blue/black color. Even so, better vision is frequently mentioned as bilberry's primary advantage. Bilberries are mostly known to most people due to their purported advantages for vision, particularly at night. A 2-year study found that taking 120 mg of bilberry anthocyanins daily enhanced visual function by around 30% in patients with glaucoma, a condition that leads to progressive blindness; the placebo group had a decline in visual function. Nine different batches (F1–F9) of bilberry-loaded Nanoparticulate In-situ gel were produced using isotropic gelation, and they were then homogenized to reduce size. The formulation F3 (two grams of calcium chloride and three grams of sodium alginate) had the highest encapsulation effectiveness (EE%) of the resulting Nanoparticles, measuring 92.33±0.38. The efficacy of glaucoma treatment was evaluated using a particular batch (F3) in combination with chitosan, HPMC, and Pluronic F127 gel. It was discovered that the drug release for formulations F1–F3 was 82.11%, 88.12%, and 94.0 %, respectively.

5. Conflict of interest

The authors have no conflict of interest.

6. Acknowledgement

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