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Phytopharmaceutical Delivery of Rubia Cordifolia Herbosomal Gel Through Skin-Formulation, Characterization, and Quality Assessment

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KEYWORDS

Herbosome, Rubia cordifolia, manjishtha,phytomolecules, carbopol940,validation, Phosphatidylcholine, Soyalecithine.

ABSTRACT:

Background: The novel herbosomal technology helps to enhance the bioactivity and targeting of plant extracts to the skin and connect the NDDS system and the traditional system. Herbosome is a kind of phospholipid-based herbal nano drug delivery system with enhancement of absorption and stability profile. In this work, we have developed and designed herbosomal gel containing Rubia cordifolia (RC) extract (Manjistha) with more disintegration and dissolution, and extended release.

Methods: RC (Manjistha) is a traditional medicine, soluble in water but poor absorption is the main limitation, which reduces its bioavailability and efficacy. RC extract herbosomes were successfully developed using Phosphatidylcholine and Soyalecithine using the solvent evaporation method. Carbopol 940 was found to be best gelling agent for the preparation of gel. Evaluation studies were carried out in extract and gel formulations like Entrapment or absorption efficiency, drug content, spreadability, pH, and viscosity.

Results: The optimized batch of *Rubia cordifolia* shows the lowest particle size of 98.7nm, the highest drug loading of 98.62%, and the highest Entrapment efficiency of 98.12%, No drug-excipient interaction was observed in FTIR. Percent cumulative release of herbosome showed release (78.23 ± 0.045% in 8 h) in pH 7.4 phosphate buffer. Herbosomal gel with 1.5% Carbopol 940 showed good consistency, viscosity, and spreadability, and the pH found was 6.39 ± 0.03. Gel remained stable at 25 °C for a week of study.

Conclusion: From the overall, experimental data. It has been suggested that RC-Herbosomal gel developed with the phospholipid-coated vesicular system can be considered a promising and targeted delivery system to enhance the solubility and permeability of the blood-brain barrier (BBB). Phospholipid also protects the drug from degradation from P-glycoprotein, cytochrome P-450, and cytochrome 3A4. Protection from these enzymes enhances bioavailability and thus absorption.

Introduction

Nowadays herbal formulations are gaining favorable importance for the treatment of many diseases due to their targeted significance effect and lesser side effects as compared to allopathic medicines.[1] There are several drugs of synthetic or natural origin showing low

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solubility and bioavailability. The reasons for low bioavailability are the presence of a *Permeabilty-Glycoprotein pump* effluxing the drug back to the lumen, the presence of liver and intestinal metabolizing enzymes, and pH-mediated degradation. Thus, desired nanocarriers play an important role in delivering drugs in systemic circulation. The phospholipid-drug complex could be the promising carrier, in which the absorption process is similar to the process through which the triglycerides and essential phospholipids are absorbed. It has been found that the mechanism for absorption of the phospholipid-drug complex is similar to that of the endogenous absorption of phospholipids through enterocytes. The phospholipid showed two fatty acid chains attached to the glycerol (diacylglycerol) in its structure, after hydrolysis, it releases fatty acid, which affects absorption. [2,3] By using herbasomal drug delivery-based technology it is possible to improve phytopharmaceuticals' bioavailability of herbal extracts for medicinal application.[4], also plant extracts can be easily standardized accordingly and may be formulated as phytosomes for a systematic investigation. Phytosomal herbal formulations could be better absorbed, and as a result, it enhances bioavailability than conventional phytomolecules or botanical extract.[5]. This study is conducted to formulate the herbosomal gel using, manjishtha extract. *Rubia cordifolia Linn*. commonly known as Indian Maddar or manjishtha. The roots and stems are known sources of Anthraquinones, the roots are antioxidant, anti-inflammatory, anticancer, immunomodulator, and hepatoprotective and are extensively used against blood, urinary, and skin diseases.[5,6] The aqueous extract of RC is known for its anti-inflammatory activity in rats.[6] It has been reported that *Rubimallin* is a well-known component found for anti-inflammatory action. Many studies were performed using the aqueous extract for anti-inflammatory activity in Wistar rats with carrageenan paw edema model in a dose-dependent manner, which is comparable to that of phenylbutazone.[7] *Rubimallin* also inhibits the lipoxygenase enzyme pathway, responsible for the catalysis of the production of many inflammatory mediators such as leukotrienes that cause asthma, arthritis, and other inflammatory disorders and the production of cumene hydroperoxides.[8] RC also showed nitric oxide scavenging activity.[9] Thus indirectly it can be used as an antioxidant. Munjistin is another component found in RC. A formulation containing munjistin showed antiproliferative action during the rapid development of model edema.[10] This study revealed that this extract was very effective and beneficial in the treatment of inflammation in the form of herbal gel. Therefore, the purpose of the present study was to formulate the herbosomal gel of manjistha extract and to evaluate the physicochemical properties in terms of pH, viscosity homogeneity, and spreadability of the herbosomal gel.

2. Material and methods

Materials

Manjistha extract was purchased from Amruta Herbal Pvt. Ltd. Soyalecithin was purchased from Shiva Laboratories**.** Vanillic acid (marker) was purchased from Innovative Chemicals, Mumbai The other materials like Triethanolamine**,** Dichloromethane, and Carbopol 940 were procured from S.D. Fine chemicals, Mumbai.

3. Saturated solubility studies

The study aimed to assess the drug's solubility in excipients, and solvents using the test tube method. For excipients, (1g) was transferred to a test tube The extract was added incrementally until completely dissolved, the lesser the amount of excipient required to solubilize the extract more will be the solubility. Soya lecithin, phosphatidylcholine, and phosphatidylserine were used, observing the minimum phospholipid needed to dissolve the extract in its melted state. After cooling, the presence of the crystal was visually checked. The Soyalecithin requiring the least amount to dissolve the drug was chosen for formulations. The study concluded upon achieving a clear solution of molten lipids with phosphatidylcholine. Thus it was selected as a model excipient for further studies.[11]

4. Preparation of trial batches

Preliminary studies were performed to determine the factors such as the effect of rotation speed, time, and temperature on the particle size of herbosome which will affect the formulation process. The Herbosomes were formulated using reflux followed by solvent evaporation technique. Phytosomes were prepared in different ratios, i.e. 0.5:1, 1:1, 1:2, and 1:3 of extracts to a combination of Soya lecithin and phosphatidylcholine. Soya lecithin and extracts were dissolved in dichloromethane. The

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solution was mixed and poured into a 200 ml round-bottomed flask. The mixture was refluxed for different durations i.e., 1-2 hours and at a temperature not exceeding 60°C. And then after the formation of a thin layer phosphate buffer was added. The resulting solution was evaporated, and dried under vacuum (40°C); the residues were gathered and stored in desiccators for further use.[12]

5. Optimization of herbosomal formulation

3^2 factorial design was used in the formulation of herbosome using Design Expert software version 13, where two factors were examined at three possible levels. Nine combinations were obtained through software. Soya lecithin, phosphatidylcholine (X_1), and Dichloromethane (X_2) were taken as the independent variables while the dependent variables were the particle size (Y_1), %Drug content(Y_2) and entrapment efficiency (EE) (Y_3). The specific details of the experimental design and the resulting formulations are presented in Table 1.

Table 1. Variables in 3^2 designs for the preparation of herbosomes

Independent variables	Levels used, actual (coded factor)		
	Low level (-1)	Medium level (0)	High level (+1)
X_1 = Soyalecithin + phosphotidylcholine	500	1000	1500
X_2= Dichloromethane	10	15	20
Dependent variables	Constraints		
Y_1 = Particle size (nm)	Optimum (100–200 nm)		
Y_2= %Drug content	Maximum		
Y_3 = Entrapment efficiency	Maximum		

6. Characterization and Evaluation of Herbosomes

6.1 . Particle size and zeta potential measurement

For finding average particle size and polydispersity index of herbosomes photon correlation spectroscopy using SZ-100 equipment from *HORIBA Ltd., Tokyo, Japan,* at a temperature of 25°C, using scattering angle of 90°. Before measurement of particle size colloidal dispersion was diluted at a concentration of 10% using filtered double distilled water.[11] The polydispersity index was investigated to assess the uniformity of the particle size distribution. Furthermore, the surface charge of the drug-loaded dispersion was measured using a zeta potential analyzer, specifically the (SZ-100 from HORIBA Ltd).

6.2. % EE and drug loading

The entrapment efficiency of prepared herbosomes was calculated by the centrifugation method. About 2 ml of dispersion and 5 ml of methanol were mixed and centrifuged in a cooling centrifuge (REMI-C24 BL. Remi Eletrotechnik Ltd., Mumbai, India) for 30 min at 3,000 rpm. The supernatant was diluted with the appropriate solvent and analyzed using a UV-VIS Spectrophotometer (V-1800, Shimadzu, Japan) at a wavelength of 340 nm.[14] The drug loading (% DL) was the weight of the encapsulated drug divided by the weight of the lipid (w/w). EE and drug loading were calculated by Equations (1) and (2), respectively. [14,15]. The entrapment efficiency (EE) is calculated by using the following formula:

$$\%EE = A-B/A \times 100\% \quad (1)$$

$$\%DL = C/A+D\times 100\% \quad (2)$$

A = The amount of extract added

B = The amount of extract in the supernatant

C = The amount of extract added

(DL) D = The number of phospholipids added

6.3. % Drug Content

The drug content was determined by dissolving the 1ml of herbosomal suspension containing dispersed herbosomes in methanol to break the herbosomal structure and release the entrapped drug. After suitable dilution with were analyzed spectrophotometrically using a UV- UV-visible spectrophotometer (Shimadzu, UV 1800) at a wavelength of 254 nm and drug content was determined.[14], and is calculated by using formula.

%Drug content = amount of drug found/ Total weight of dosage form x100 (3)

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7. Freeze-drying

After preliminary characterization, the lipid dispersion underwent lyophilization using the (Martin Christ ALPHA1-2LD Freeze Dryer), with a capacity of 4.5 L, and a condenser temperature set at -70°C for 24 hours to assess the crystallinity of the formulated herbosomes. This process aimed to transform the liquid into a dried state. Samples were subjected to freeze-drying under a pressure below 0.5 m Bar and a temperature around -50 ºC. To prevent lysis during storage, a cryoprotectant solution of 3% Mannitol was introduced into the dispersion, and the samples were stored at – 4 °C for further investigation.

8. FTIR spectroscopy

The freeze-dried herbosomes underwent Fourier Transform Infrared (FTIR) spectroscopy investigations using a (Perkin Elmer Spectrum BX instrument from the United States). This analysis aimed to examine potential interactions between the drug and phospholipids in the mixtures. The sample preparation utilized the potassium bromide (KBr) disk method.

9. Scanning Electron Microscopy

The surface morphology of freeze-dried optimized formulation was studied using scanning electron microscopy (SEM) (FEI, Quanta 200, Netherland) at a magnification range from 30 x to 1 lac x, and Resolution set was 3.5 nm, High Voltage – 30 Kv using (Tungsten Filament as a source, and Software xT microscope Control). Lyophilized powder of optimized formulation was dusted onto double-sided tape on an aluminum stub and coated with gold using a cold sputter coater in an SEM chamber to a thickness of 400 Å, and then photomicrographs were captured by operating at an accelerating voltage of 15 kV electron beam.

10. In vitro release of herbosomes

In vitro, drug release study from extract and optimized batch of herbosomes pH 7.4 phosphate buffer to ensure the controlled release. Dialysis membrane with a pore size of 2.4 nm and molecular weight cut off of 12,000-14,000 kDa was soaked in double-distilled water for 12 h before use. Four milliliters of dispersion were poured into the bag with the two ends fixed by clamps. The bags were placed in a conical flask containing 50 ml of the above-mentioned buffers at 37 ± 2°C as a dissolution medium. The conical flasks were placed into a thermostatic shaker (BM2-262, Biomedica, India) at a rate of 140 times per minute. At 0.5, 1, 2, 4, 6, 8, 12, and 24 h, 5 ml aliquots were removed and were replaced with fresh dialysis medium at the same temperature. Samples were analyzed by using a UV-visible spectrophotometer at 254 nm. All the operations were carried out in triplicate. [18,19]

11. Stability studies of herbosomes

Long-term stability studies of optimized formulation were evaluated as per ICH guidelines Q1 A(R2) (2003). (5 ml) was transferred in amber-colored glass vials, sealed, and stored upright in a stability chamber. Physical and chemical stability was evaluated for twelve months by storing them at 30 ± 2°C/65 ± 5% RH. Samples were withdrawn at specified time intervals (0, 3, 6, 9, and 12 months of storage) and assessed for changes in particle size, PI, and Entrapment efficiency in addition to physical appearance and ease of reconstitution.[16]

12.Formulation of herbosomal gel

12.1 Preparation of Gel using carbopol

Carbopol resin was dispersed in glycerol. The mixture was stirred until it gets thick followed by neutralization by dropwise addition of TEA. TEA was added until transparent gel appeared. Different batches were prepared with different concentrations of polymer herbosome incorporation. A batch was selected for further studies which showed good viscosity and hydration of water into gel as compared to different concentrations.[17] as indicated in Table 2.

Table 2. Preparation of Carbopol Gel

Batches	Carbopol 940	Glycerin	TEA	Water
A1	0.5%	10%	1%	Q.S.
A2	1.0%	10%	1%	Q.S.
A3	1.5%	10%	1%	Q.S.
A4	1.8%	10%	1%	Q.S.

12.2. Incorporation of Herbosome:

The herbosome-containing drug was mixed into different concentrations of Carbopol gel by an electrical mixer 25 rpm/2 min, with the concentration of herbosome in hydrogel being 1% (w/w suspension/total)

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12.3. Physicochemical evaluation of herbosomal gel

The herbosomes enriched hydrogel were characterized for their physicochemical properties such as color, odor and pH, viscosity, and drug content.

12.4.Measurement of pH

The pH of various gel formulations was determined by using a digital pH meter. One gram of gelwas dissolved in 100 ml distilled water and stored for two hours. The measurement of the pH of each formulation was done in triplicate and average values were calculated. [17]

12.5. Viscosity

The viscosity of the formulation was determined by CCT_8_600110 using RST-CC Brookfield Rheometer at 100 rpm [17]

12.6. Spreadability

The spreadability test is commonly performed to evaluate the ease with which a gel or cream formulation can be spread over a surface. The gel sample should be ensured at room temperature before testing. A sufficient amount of gel was used to cover the surface area of the glass plate. Gently gel was spread over the surface of the glass plate consistently, exerting a uniform pressure. The timer was started simultaneously by applying the gel onto the glass plate. After a predetermined time interval of 30 sec the diameter of the gel spread was measured using vernier calipers or a ruler. Measure both the maximum diameter and the diameter perpendicular to it to calculate the average spread diameter. spread diameter in millimeters (mm) was recorded. The spreadability of the gel formulation was determined, by measuring the diameter of 1 gm gel between horizontal plates (20×20 cm^2) after 30 sec. The standardized weight tied on the upper plate was 125 gm. T is calculated by using a formula. [17]

$$S = M \times L / T \times A, \quad (4)$$

Where,

M = Mass of the gel applied (in grams)

L = length of the glass plate(in centimeters)

T = Time taken to spread (in seconds)

An area covered by gel(in square centimeters)

13. Stability Studies of Herbosomal Gel

The optimized formulation of herbosomal gel was selected for the stability study. Two other formulations were made as control which was A2 & A4. A2 was formulated without the addition of propylparaben as a stabilizer and A4 was formulated without the presence of extracts as active pharmaceutical ingredients. Stability studies were performed on these three formulations by keeping them at three different temperatures, i.e. 45 ± 2˚C, 25 ± 2℃ and 4 ± 2˚C for 21 days.[17] Parameters observed for the herbosomal gel are the determination of the pH value, viscosity, homogeneity, and spreadability of the herbosomal gel.

13.1. Centrifugation Test

10g of herbosomal gel was added to a centrifuge tube. During centrifugation, the gel was subjected to a cycle of 3000 rpm for 30 minutes at room temperature. Centrifugation was performed by Model Eppendorf Centrifuge 5820 R. [20,21]

14. Development and Validation

14.1. UV spectroscopy

14.2. *Spectroscopic Studies*

Selection of solvent:

After solubility studies were performed in various solvents, the UV spectra of the extract were recorded. maximum absorbance was observed in water hence water was selected for further studies.

Determination of λ_{max}

100µg/ml solution of manjistha extract and vanillic acid(marker) was prepared in distilled water. The UV spectrum was recorded in the range of 200-400nm at (Shimadzu UV- visible spectrophotometer (UV- 1800)). 254 nm and 240 nm λ_{max} were recorded respectively.

Standard Calibration Curve of vanillic acid

10mg extract dissolved in 10 ml of distilled water. From this stock solution, 1 ml was withdrawn and diluted to 10 ml of distilled water. From this primary solution, serial dilutions were made by withdrawing 1,2,3,4,5,6,7 ml and diluting to 10 ml with distilled water to obtain the solutions in concentrations of 10,20,30,40,50µg/ml. The absorbance was measured at 240 nm using a UV Spectrophotometer.

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UV Standard Calibration Curve of Manjistha extract

10mg of extract was added in 10 ml of distilled water. From this stock solution, 1 ml was withdrawn and diluted to 10 ml of distilled water. From this primary solution, serial dilutions were made by withdrawing 1,2,3,4,5,6,7 ml and diluting to 10 ml with distilled water to obtain the solutions in concentrations of 10,20,30,40,50 µg/ml. The absorbance was measured at 254 nm using a UV Spectrophotometer.

Validation parameters of herbosomal gel

Precision

The precision of the method was checked by carrying out repeatability of measurement, intraday, and interday precision. 20 µg/ml solution was used to check the repeatability, and peak amplitude of derivative spectra of the standard solution. Analysis was performed nine times and % RSD was calculated. Intraday precision was carried out by repeated measurements of the peak amplitude of derivative spectra of standard solutions ie 20 µg/ml. at the same concentration levels three times on the same day. Interday precision was studied by comparing the results on three different days analyzing 20 µg/ml. of solution.

Accuracy

To validate the accuracy of the method for quantifying, manjishtha extract gel, standard addition and recovery tests were conducted. Recovery studies involved adding three different concentration levels (80%, 100%, and 120% of the target concentration of 50 µg/ml) in triplicates. Distilled water was used to adjust the volume of diluted samples. The percentage of recovery and relative standard deviation (% RSD) were calculated, yielding values below 2%. Peak areas from the samples were compared to those of a standard solution, and the percentage recovery of the total drug was determined using a specific formula. The mean percentage recovery ± standard deviation (S.D.) was expressed for each concentration level.

LOD and LOQ

The limit of detection and limit of quantification of extract was calculated and mentioned using the following equation as per ICH

$$LOD = 3.3 \times \sigma/S \quad (5)$$

$$LOQ = 10 \times \sigma/S \quad (6)$$

Where, σ = Standard deviation of the response S = Slope of the calibration curve.

High-Performance Liquid Chromatography Of marker (Vanillic acid)

The HPLC system employed for analysis was the Thermo–Ultimate 3000 High-performance liquid chromatography (HPLC) system manufactured by Thermofisher Scientific. This system included an isocratic or quaternary solvent delivery module, a gradient solvent delivery module, an online degasser, a column thermostat, an autosampler, and a UV detector, all controlled by Chromaleon software. A Hypersil C18 HPLC column with dimensions of 250 mm length, 4.6 mm inner diameter, and 5 mm packing filled with octadecyl silane stationary phase was used, with the column temperature set at 25 °C. The flow rate was kept at 1.0 ml/min at 240 nm at a run time of 10 min.

Mobile Phase: Distilled water, methanol, and acetic acid were mixed at a ratio of (700:300:10) ml respectively followed by passing through a nylon membrane filter having a pore size of 0.45µm and sonicate to degas.[30]

HPLC studies were performed in 20µg/ml vanillic acid solution at a wavelength of 240 nm and 20µg/ml manjishtha extract and gel at a wavelength of 254 nm. The presence of vanillic acid at the same retention time in manjishtha extract confirms anti-inflammatory activity[31].

1. Result and Discussion

1.Saturated solubility studies

The kind and concentration of surfactant, along with other formulation parameters including phospho lipid type and concentration, have a major impact on the particle size polydispersity and entrapment efficiency of liquid nanoparticles. A variety of phospholipids with varied chain lengths were assessed since choosing the right lipid core is crucial to producing stable herbosomes. The perfect surfactant with proper concentration is primarily responsible for keeping the nanoparticles stable in the colloidal state and preventing the development of the particle size while being stored.

Mangistha extract is poorly water soluble showing high lipophilicity. Thus out of phosphatidylcholine and

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phosphatidylserine, phosphatidylcholine exhibited maximum solubility in extract. Thus it was selected for further studies.

2.Preparation of trial batches

Depending on the obtained result of saturation solubility studies in various phospholipids was further used in the preparation of trial batches Table 3(A), and the final combination of lipid and surfactant was chosen based on its stability, particle size, and zeta potential. The batches of herbosomes were prepared by a solvent evaporation method using a rotary evaporator. The combination of phosphatidylcholine and extract showed less particle size (98.7 nm), higher Polydispersibility index (0.8 nm), and zeta potential (-30.6 mV). Table Table 3(B)indicates different factors influencing herbosomes.

Trial batches of Manjistha extract and soya lecithin were taken in different ratios. Then by changing with parameters (Time, RPM) for the formulation of herbosomes., After microscopic observation, it was found that the Batch (F3) 1:1 ratio shows a clear and spherical shape. So this was selected for further studies. The microscopic image of the optimized formulation is indicated in Figure 2.

Table 3 (A). Formulation of Trial Batches of Herbosomes

Ingredients	Trial batches of extract		
	T1	T2	T3
Mangistha extract (mg)	500	500	500
Phosphotidylcholine (mg)	500	1000	1500
Dichoromethane(ml)	10	15	20

Table 3 (B). Experimental values of different factors influencing herbosomes

Sr no.	Batch	Rotary evaporator time (min)	RPM	Temperature (^{0}c)	Shape
1	**F1**	45	80	60	Irregular
2	**F2**	40	120	60	Irregular
3	**F3**	60	140	60	Regular and Spherical
4	**F4**	45	100	60	Irregular
5	**F5**	45	120	60	Irregular
6	**F6**	60	140	60	Irregular
7	**F7**	45	140	60	Irregular
8	**F8**	60	80	60	Irregular
9	**F9**	60	120	60	Irregular

3. Evaluation of Herbosomes

Particle size and zeta potential (ζ) measurement and polydispersity index (PDI)

Particle size is an essential characteristic because it determines the stability of the nanoparticle system. Smaller particle size increases the surface area and, consequently, leads to high solubility, making the particles easier to absorb in the body. [22], and zeta potential is the method for figuring out the surface charge of nanoparticles in a colloidal solution. A thin layer of counter ions is drawn to the surface of nanoparticles due to their surface charge (Stern layer). The nanoparticle diffuses throughout the fluid accompanied by this double layer of ions. The zeta potential of the particles, which is the electric potential at the double-layer interface, usually ranges from +100 to -100 mV. Agglomeration of the zeta potential value is ultimately caused by hydrogen bonding and interparticle interactions such as van der Waals and hydrophobic interactions. [16] The results of particle size analysis of all nine batches are shown in Table 4. Amongst all, the (F3) batch showed the lowest hydrodynamic particle size (Zavg) of 98.7 nm. The graphical presentation of the particle size distribution of F3 is depicted in Figure 1(A). Zeta potential was found to be – 30.6 mV Figure 1(B). A negative value is due to the carboxylic group of lipids and the insufficiency of counter ions for neutralization within the electrical double diffuse layer. Values of zeta potential showed prepared F3 has sufficient charge and mobility to inhibit

the aggregation of particles. It has been reported, that in a combined electrostatic and sterical stabilization, a zeta potential of about -30.6 mV is good for physical stability.[16]. Hence F3 has excellent physical stability as they have a zeta potential of -30.6 mV. [16, 23] The polydispersity index represents the distribution of nanoparticles in a dosage form. In this study, the polydispersity index was found to be 0.8 nm for monodispersed particles. Based on the results of the particle size analyzer Table 4, Monodisperse nanoparticles can improve the stability of the nanoparticle system because they show the size, shape, and weight of homogeneous particles. A higher polydispersity index means that more particles are aggregated, or in other words, the preparation is increasingly unstable.[2]

Table 4. %Drug Entrapment Efficiency and %Drug Content of Manjisthaextract

Factors					Responses		
Sr. No.	Batch Code	Extract	PC X_1	DCM X_2	P.S	%DC	%EE
1	F1	500	500	10	159	89.0	89.8
2	F2	500	500	15	210	83.4	80.30
3	F3	500	500	20	98.7	98.62	98.12
4	F4	500	1000	10	110.2	97.4	96.80
5	F5	500	1000	15	112.1	90.82	91.67
6	F6	500	1000	20	211.1	89.04	91.05
7	F7	500	1500	10	115.1	91.11	92.15
8	F8	500	1500	15	112.7	95.45	93.77
9	F9	500	1500	20	102.3	96.7	97.17

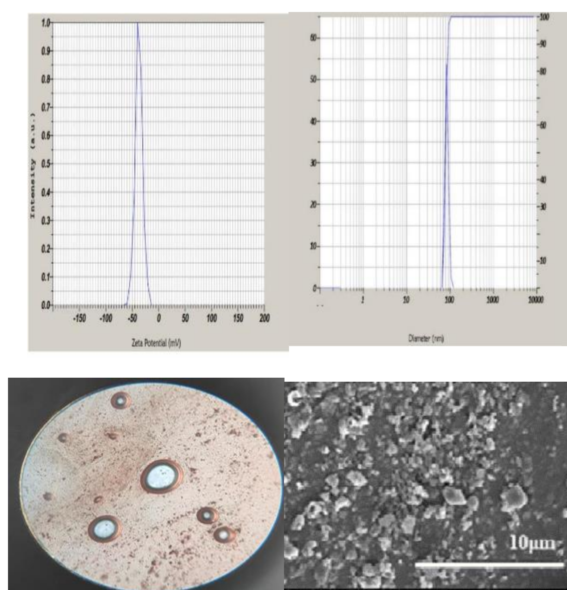

Figure 1 (A) Particle size of optimized batch herbosomes (B) zeta potential (C) Microplate picture (D) SEM % EE and drug loading

Entrapment efficiency determines how much percentage of the active substance is absorbed in the herbosomes compared to the total extract added. The absorbance values showed that the concentration of the extract was absorbed, while the entrapment efficiency of the herbosomes was identified using a linear equation of the standard extract curve. Thus higher the entrapment efficiency greater the percentage of drug release.[25], which is associated with the drug's lipophilic nature, indicating a stronger affinity for the lipid matrix. The extract was non-lipophilic and insoluble but after herbosomal formulation with the increase of entrapment efficiency, it was concluded that the extract was found to be lipophilic and solubility was increased as well. The detailed results of % EE for all batches are provided in Table 4. Among the batches, the F3 batch showed the highest % EE at 98.12% and the smallest particle size of 97.6 nm, establishing it as the optimized batch. The consistently high values of EE% in this study suggest that the chosen phospholipid and surfactant compositions effectively facilitated the entrapment of extract in the

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herbosomes. The drug % content was found for the F3 batch 98.7%. Details of the result as outlined in Table 4.

4. Effect of formulation variables on particle size of nanoparticles

The results of the experimental design were statistically analyzed using Design Expert software (version 13.0, Stat-Ease Inc., Minneapolis, MN).

Observed responses of nine formulations were fitted to various models by using software 2FI model were best-fitted for all three responses, i.e. hydrodynamic particle size (Z_{avg}) and % EE and % DC.

Particle size(Y_1) = $+507.33-0.243431X_1-23.89297 X_2+0.014860X_1X_2$ (7)

where,

Y_1= particle size

X_1= Phosphatidylcholine

X_2= Dichloromethane

%Entrapment Efficiency(Y_3) = $+44.20649+0.034890 X_1 +3.02272 X_2-0.002065 X_1X_2$ (8)

where,

Y_2 = % EE

X_1= Phosphatidylcholine

X_2= Dichloromethane

% Drug Content (Y_2) = $+48.27614+0.033249X_1 + 2.74192 X_2 -0.001944 X_1X_2$ (9)

Y_3 = % DC

X_1= Phosphatidylcholine

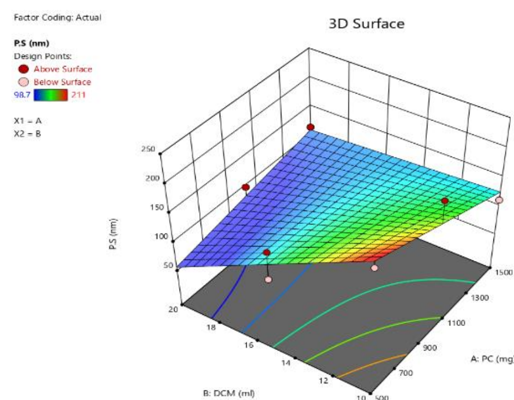
X_2= Dicholoromethane

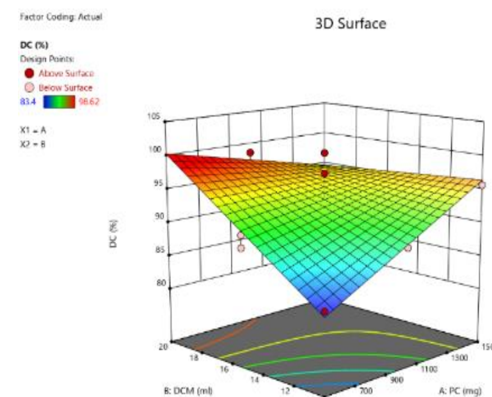
Response variables Y_1 and Y_2 were fitted with a 2FI model. A synergistic effect of the factors on the response is represented by the positive sign, whereas the antagonist relationship is represented by the negative sign.

Since the values of r^2 are quite high for both the responses (0.9496 for particle size and 0.9869 for %EE), the polynomial equations form an excellent fit for the experimental data and are highly statistically valid Analysis of variance (ANOVA) for response surface quadratic model Y_1 (Particle size, Z_{avg} in nm) and Y_2 (% EE) shows that the values of "Prob4F" are less than 0.05 which indicates model terms are significant.

Response surface plots

Response surface plots are significant three-dimensional surface curves for understanding the interaction patterns. Three-dimensional response surface plots were generated at different levels by the Design-Expert software (Figures 2(A) and 2(B) 2(C) for the studied responses, that is, particle size (Y_1), and EE% (Y_2), and % DC. The results indicate that the amount of soya lecithin (X_1) has a positive influence on the particle size and DCM (X_2) shows a negative influence on particle size. (Figure 1 (A). (Figure 2(A) showed that phospholipids and DCM have a positive effect on %EE, Whereas in the case of %, DC Figure 2(C) showed that both factor phospholipids and DCM showed a positive effect on response % DC.





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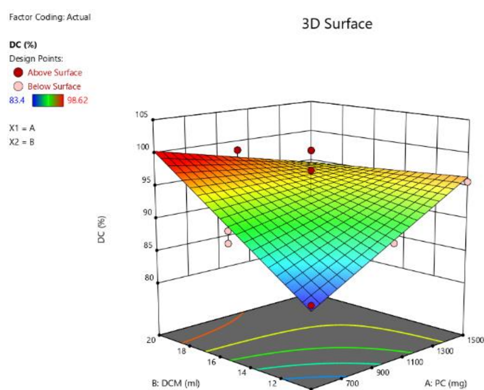

Figure 2. Response surface plots: (A) particle size (Z_{avg}) and (B)% EE (C) %D.L (D)Herbosomal Gel

5. Fourier transform infrared spectroscopy (FTIR)

The characteristic FTIR spectra of extract, soya lecithin,herbosomes, and gel are shown in (Figures 3 A, B, C, and D)All details shown in Tables 5(A), 5(B), 5(C), 5(D). The possible interaction between the Extract and Soyalecithin was studied by FTIR spectroscopy. There was no considerable change in the positions of characteristic absorption bands and bonds of various functional groups present in the Extract. This observation suggests that the Herbosomal gel shows no prominent change in its characteristics even in its physical mixture. The results of FTIR spectra indicated no interaction between Extract and Soyalecithin. It showed that Manjistha Extract was compatible with Excipients.[19]

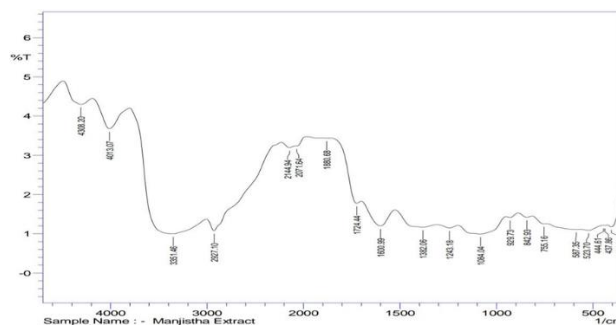

Figure 3(A).FTIR spectra of manjistha extract

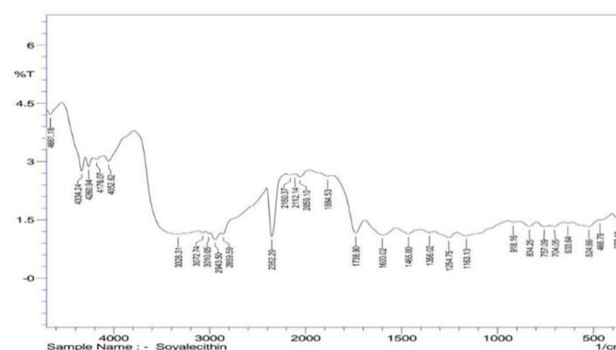

Figure 3(B).FTIR spectra of soya lecithin

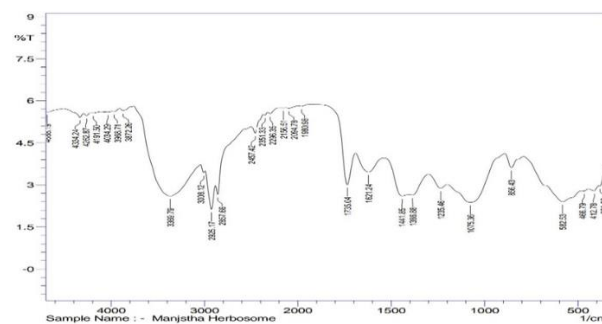

Figure 3(C).FTIR spectra of herbosomes

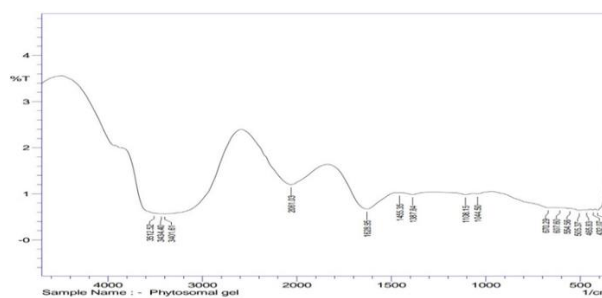

Figure 3(D).FTIR spectra of herbosomal gel

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Table 5 (A). Functional Group and their Frequencies of FT-IR Spectra

Groups	ObservedPeak	Principle Peak
=C-H (Bending)	755.16	675 - 1000 cm-1
C-O(Stretching)	1084.04	1000 – 1300 cm-1
C-O(Stretching)	1243.18	1050-1150 cm-1
C=O(Stretching)	1724.44	1670-1820cm-1
C-H (Bending)	1382.06	1350 –1480 cm-1
O-H(Stretching)	3351.46	3200 –3600 cm-1
C-H(Stretching)	2927.10	2850 - 3000cm-1

Table 5(B). Functional Group and their Frequencies of FT-IR spectra of Soyalecithin

Groups	Observed Peak	Principle Peak
C – H (Stretching)	2943.5	2850 – 3000 cm^{-1}
C = C (Stretching)	1465	1400 – 1600 cm^{-1}
C = O (Stretching)	1738.90	1670 – 1820 cm^{-1}
C – O (Stretching)	1163.13	1000 – 1300 cm^{-1}

Table 5(C). Functional Group and their Frequencies of FT-IR spectra of herbosomes

Groups	Observed Peak	Principle Peak
=C–H (Bending)	856.43	675- 1000 cm^{-1}
O-H (Alcohol)	3369.79	3200- 3600 cm^{-1}
C-O (Stretching)	1235.46	1000- 1300 cm^{-1}
C–H(Stretching)	2926.17	2850- 3000 cm^{-1}
C-H(Stretching)	3008.12	3000- 3100 cm^{-1}

Table 5(D). Functional Group and their Frequencies of FT-IR spectra of herbosomal gel

Groups	Observed Peak	Principle Peak
C – O (Stretching)	1108.15	1050-1150 cm^{-1}
C – O (Stretching)	1044.50	1000-1300 cm^{-1}
C = C (Stretching)	1455.35	1400-1600 cm^{-1}
C = C (Stretching)	1628.95	1620-1680 cm^{-1}
O – H (Stretching)	3401.61	3200-3600 cm^{-1}

6. SEM of optimized batch

The optimized lyophilized sample (F3) was examined by SEM to analyze the shape and surface morphology of the designed herbosomes. All herbosomes appeared to be spherical with uneven surfaces Figure 1(C).

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7. In vitro drug release study

Percent cumulative release of batches A1 to A4 was performed in a 7.4 pH buffer using a dialysis membrane. It was observed that out of all 4 batches A1 batch shows 78.23% release up to 8 hrs. Thus experiment revealed that herbosomes are more promising formulations. The release studies data are shown graphically as cumulative % drug release-time (h) in (Figure 4).

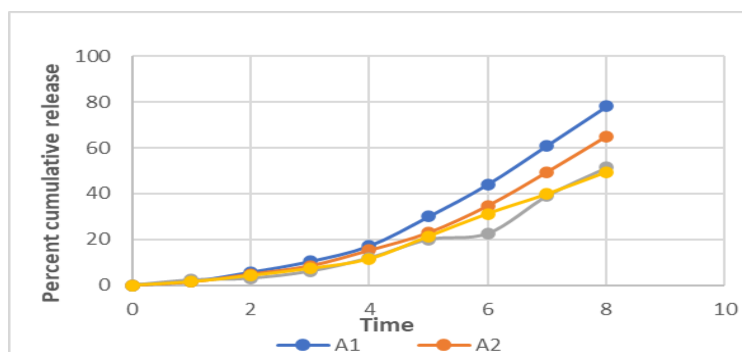

Figure 4. In vitro drug release profile of herbosome batches from A1 to A4 in 7.4 pH buffer using Dialysis bag method in 50 ml Round bottom flask at Magnetic Stirrer.

8. Stability studies of herbosomes

The findings concerning the stability of herbosomes are outlined in Table 6. Upon freeze-drying, The reconstituted mixture exhibited a uniform dispersion without any indications of settling, lump formation, or drug precipitation throughout the study period. Noteworthy, there were no significant alterations observed in the assessed parameters[27]. In conclusion, the results from the stability study indicate that the formulation maintained its stability over the designated duration of the study.

Table 6. Long term stability studies of herbosomes

Parameter	Testing points				
	0 day	*3rd month*	*6th month*	*9th month*	*12th month*
Color	*reddish brown*	*reddish brown*	*reddish brown*	*reddish brown*	*reddish brown*
Appearance	Homogeneous dispersion with no sign of sedimentation	Homogeneous dispersion with no sign of sedimentation	Homogeneous dispersion with no sign of sedimentation	Homogeneous dispersion with no sign of sedimentation	Homogeneous dispersion with no sign of sedimentation
Particle size	119± 4 nm	166.4±3 nm	170 ±3 nm	182.4±6 nm	199.3±5 nm
Entrapment efficiency	98.32±0.2%	96.76±1.2%	94.84±0.2%	91.39±0.2%	90.12±0.2%
Zeta Potential	- 60.8 Mv	-48.4 pmV	- 40.8 mV	-39.1 mV	-39.8 mV
Polydispersibility index	0.342	0.546	0.222	0.418	0.416

9. Formulation of herbosomal gel

Herbosomes are made by reacting extract with soy lecithin dissolved in DCM. After solubilization was completed, the complex compounds formed were removed by solvent evaporation technique. [1] Herbosomal gel was prepared by using Carbopol 940 as the gelling agent as shown in Table 7 and Figure 2(D) [22].

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Table 7. Optimization of Carbopol concentration

Batches.	Carbopol 940	Glycerin	Triethanolamine	Water	Result
A1	0.50%	10%	1%	Q.S	Good Consistency
A2	1.00%	10%	1%	Q.S	Poor consistency
A3	1.50%	10%	1%	Q.S	Good consistency
A4	1.80%	10%	1%	Q.S	Poor consistency

10. Physicochemical evaluation of herbosomal gel

Physical examination of gel [28]

For the physical appearance study, it was shown that the formulation with 1, 1.8% of carbopol 940 changed its color to dark brown-yellowish after one day of the gel preparation. However, with 0.5%(A1) of Carbopol 940, it remains reddish yellow which is the natural color of the extract.

Besides, this formulation (A2, A4) was a bit watery as compared to other formulations.A3 showed very good consistency with a clear appearance**.**

pH

The pH values of the herbosomal gel are found to be in the range from 6.29 ± 0.07 to 6.90 ± 0.02, which was expected since the carbopol was formulated with a pH between 5 to 5.5 and is neutralized using triethanolamine.

The pH value showed that extract gel probably would not cause skin irritation. The extracted gel was evaluated for its physical parameters such as color, odor, and homogeneity. The herbosomal gel formulation has a smooth texture and reddish yellow transparent and homogenous and it has a characteristic odor of mangishtha extract, pH (6.24- 6.45) which is good for human skin. Thus A1 and A3 batches were selected based on pH and Physical examination. Table 8 shows measured data of pH.

Table 8. Measurement of pH

Formulation code	Measured pH
A1	6.29±0.07
A2	6.75± 0.08
A3	6.39± 0.03
A4	6.90± 0.02

Viscosity

Viscosity reading of the herbosomal gel, A1, for 3 consecutive weeks at 4°C, 25°C, and 45°C storage temperatures. The viscosity of the herbosomal gel varies at different temperatures. At 4°C, A1 is gradually decreasing in viscosity reading after three weeks of study. The viscosity of A2 is not consistent every week due to the absence of a stabilizer to maintain its structure and stability which is propylparaben.

At room temperature, 25°C, herbosomal gel A1, A2, and A3 show no significant changes and is consistent throughout the three weeks of the stability study. F1 and F2 continue to show consistency when kept at 45°C after three weeks. However, A4 shows inconsistency in Generally known that viscosity is affected by the storage temperature variations, so these variations are expected.

Acceleration of chemical reactions, alteration of the activity of the active ingredients or components, viscosity, appearance, color, and odor can happen due to higher temperature [16].

The Carbopol 940 was added as a thickening gel to reach the gel requirements. The values of the viscosity were increased as the concentrations of the gelling agents were increased. Further the value between 2817.96 and 4213.42 centipoises which lies in the acceptable range[15]. for topical gel formulation indicated in Table 9(A). [15]

Table 9(A). Measurement of viscosity before and after centrifugation to check stability

Batches	Viscosity before centrifugation	Viscosityafter centrifugation
A1	3245	2532
A2	3560	3321
A3	3590	3588

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Spreadability

Higher spreadability values were indicated in the A3 batch than in any other batches. Thus A3 batch was considered as best batch. The results of spreadability are indicated in Table 10.

Table 10. Results of Spreadability

Batch code	Load applied(gm)	Initial Radius	Final Radius covered	Distance of Spreading	Time Taken (sec)	Spreadability (g.cm/s)
A1	49.34	1	2.47	1.47	60	1.2
A2	49.34	1	1.62	0.62	60	0.743
A3	49.34	1	1.34	0.34	60	1.654
A4	49.34	1	2.45	1.45	60	1.047

11. Stability testing using Centrifugation Test

During the stability study, the A3 batch herbosomal gels were subjected to a centrifugation test to identify their long-term stability. The samples were centrifuged at 3000 rpm for 30 minutes at room temperature. After the centrifugation test is done, all formulated herbosomal gels kept in every temperature show no phase separation. Physical parameters testing was done after the centrifugation test.

Centrifugation test is a direct procedure to evaluate the stability of the herbosomal gel when placed in a severe condition.[17]As There is no phase separation after the centrifugation test is done, it shows that the formulation of the herbosomal gel is stable over harsh conditions. The viscosity reading of the herbosomal gel was recorded before and after the centrifugation test.

This is to make sure there are no significant changes in the reading to maintain its stability.[16] However, the viscosity reading for A2 which was a formulation without propylparaben has significant changes after the centrifugation test. Thus, this herbosomal gel needs to have a stabilizer to prolong its stability over time (Table 9(B) indicates viscosity before and after centrifugation for observing stability.

Table 9(B). Measurement of viscosity

Batches	Viscosity at 4 °C			Viscosity at 25° C			Viscosity at 45° C		
	Week 1, 2, 3			Week 1, 2, 3			Week 1, 2, 3		
A1	2884	2845	2690	2695	3534	3075	2699	2698	2560
A2	3678	3690	4043	3560	33789	4231	3998	4032	4023
A3	3967	3620	3672	3569	3568	3567	3456	3455	3450
A4	4012	3903	3789	3590	3500	3400	3578	2217	2134

12. Development and Validation

Selection of solvent

Water was selected as a solvent due to the maximum absorbance observed.

Selection of wavelength

The maximum absorbance was observed at 240 nm for all vanillic acid concentrations, leading to its selection as the optimal wavelength for subsequent HPLC analysis. This was evident in Figure 6 which represents the linearity of vanillic acid.

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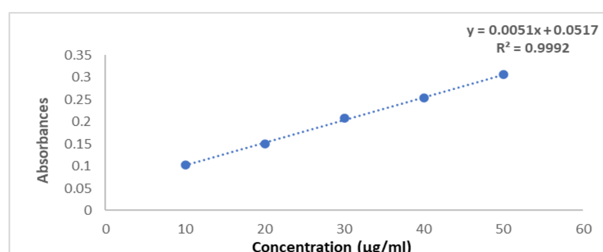

Figure 6. Mean linearity chart of vanillic acid at 240 nm.

Standard Calibration Curve of vanillic acid

Linearity

Vanillic acid was used as a marker for comparison purposes with manjishtha extract. It was quantified in quintuplicate for a concentration range of 10 to 50 µg/mL. Individual linear plots were generated for each trial, and the mean value was computed. The plotted linear regression graph showed a linear regression equation of y = 0.0051 x + 0.0517 for the mean value, with a coefficient of determination (R^2) of 0.9992. Notably, none of the consecutive R^2 values from the five linear regression plots was less than 0.999. This indicated that the optimized method was highly linear within the vanillic acid concentration range, and further experimentation was warranted. Figure 6 represents the linearity of the method via a linear graph.

Standard Calibration Curve of Manjistha extract

Manjistha extract linearity concentration was prepared in the range of 10-50 µg/ml. The regression equation was found to be Y= 0.0046 x +0.0729, and the correlation coefficient was 0.9988. A calibration curve is shown in Figure 7. The measured peak areas were plotted against corresponding concentrations to obtain a linear curve. It can be concluded from the results that the method was found to be linear.

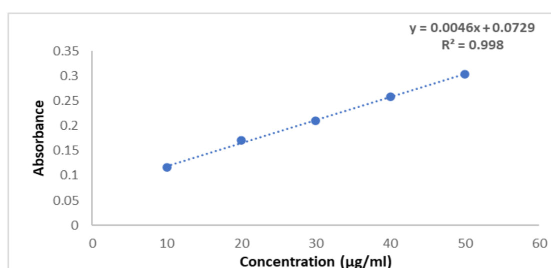

Figure 7. Standard calibration curve of manjishtha extract at 254 nm

Validation of Herbosomal gel [29]

Linearity

Manjistha extract herbosomal gel linearity concentration was prepared in the range of 10-50 µg/ml. The regression equation was found to be Y= 0.0048 x +0.0618, and the correlation coefficient was 0.9896.

A calibration curve is shown in Figure 8. The measured peak areas were plotted against corresponding concentrations to obtain a linear curve. It can be concluded from the results that the method was found to be linear.

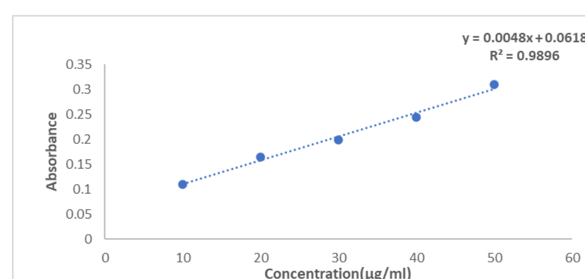

Figure 8. Linearity of manjishtha extract herbosomal gel at 254 nm

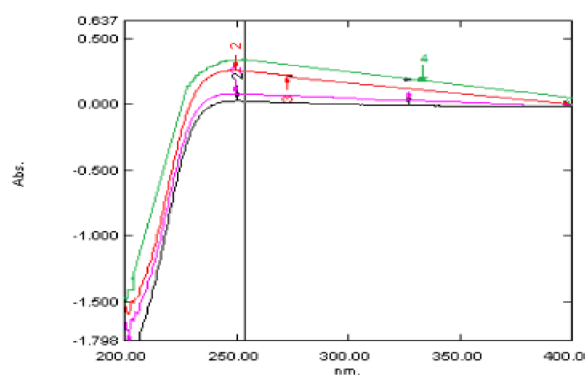

Figure 8. 1 Overlain spectra of 10-60 µg/ml concentration of herbosomal gel.

Precision

Precision studies were performed 9 times on the same day in the morning, afternoon, and evening as per ICH guideline Q2 R1. The obtained %RSD was less than 2 indicating good repeatability and low intra and inter-day variability of the developed method. Results are shown in Table 11.1 and 11.2 respectively.

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Table 11.1. Intraday precision (on the same day)

Sr no.	Concentration (µg/ml)		Absorbance	Mean	SD	% RSD
1	20	Morning	0.174	0.177	0.0032	1.80
2	20		0.180			
3	20		0.179			
4	20	Afternoon	0.176	0.176	0.0015	0.86
5	20		0.178			
6	20		0.175			
7	20	Evening	0.181	0.179	0.0015	0.85
8	20		0.178			
9	20		0.180			

Table no11.2. Interday precision (on different day)

Sr no.	Concentration (µg/ml)		Absorbance	Mean	SD	% RSD
1	20	Day 1	0.174	0.177	0.0032	1.80
2	20		0.180			
3	20		0.176			
4	20	Day 2	0.176	0.176	0.0015	0.86
5	20		0.174			
6	20		0.179			
7	20	Day 3	0.181	0.179	0.0015	0.85
8	20		0.174			
9	20		0.177			

Table 11.3. Determination of Accuracy

Sr no.	Concentration(%)	Original level (µg/ml)	Amount added (µg/ml)	Recovery (µg/ml)	% Recovery	Mean % Recovery	% RSD
1.	80	50	40	88.51	98.35	99.47	1.85%
	80	50	40	91.44	101.6		
	80	50	40	88.61	98.46		
2.	100	50	40	101.86	101.86	101.14	0.73%
	100	50	40	101.18	101.18		
	100	50	40	100.38	100.38		
3.	120	50	40	111.34	101.21	100.19	1.12%
	120	50	40	108.88	98.98		
	120	50	40	110.42	100.38		

Accuracy

The accuracy of an analytical method is the degree of agreement of test results generated by the method to the true value. It is measured by spiking known amounts of the analyte into a sample and comparing the recovered amount to the expected amount. Accuracy was determined by calculating the recovery at three levels of concentrations (80, 100, and 120%). The method was found to be accurate with a % recovery of 99.47-

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101.14% as shown in Table 11.3. which was within the acceptable criterion of 100 ± 2%.

LOQ and LOD

The LOD concentrations were found to be 2.070 µg/mL and the LOQ concentration was 5.027 µg/mL. indicating enhanced sensitivity for detection and quantification. The LOD and LOQ findings are summarized in Table 11.4.

Table 11.4. Determination of LOD and LOQ

LOD	**2.07**
LOQ	**5.027**

High-Performance Liquid Chromatography of Marker (Vanillic acid)

It was observed that the Marker i.e. vanillic acid is present in the manjistha herbosome and herbosomal gel from Figures 9, 10, and 11 respectively. Hence this showed the presence of a marker compound in finished product which shows anti-inflammatory activity for topical use.

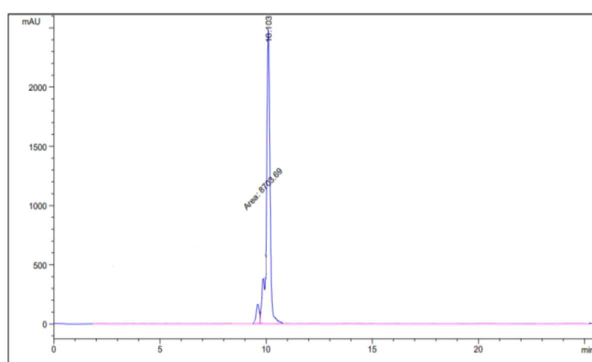

Figure 9. Chromatogram of Vanillic acid at retention time of 10.1min.

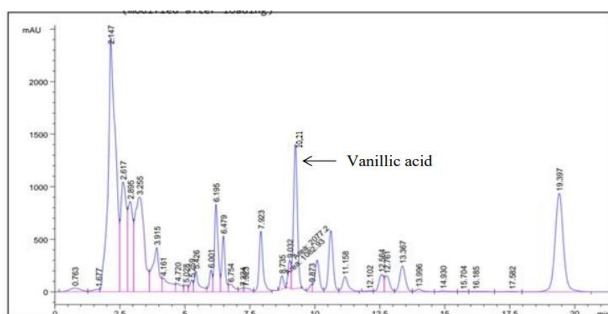

Figure 10. Peak response of Manjistha Herbosome

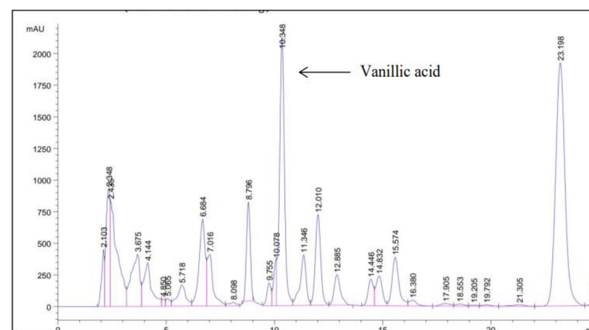

Figure11. Peak response of Herbosomal Gel

High-Performance Liquid Chromatography of Marker (Vanillic acid)

20µg/ml solution of vanillic acid was run on HPLC at 240 nm. Chromatogram appeared at a retention time of 10.1 min as indicated in Figure 9, and 20µg/ml solution of manjishtha extract and herbosomal gel. Vanillic acid was observed at a retention time of 10.21 in both extract and gel as well confirmed the presence of anti-inflammatory activity.

Conclusion

The formulation of herbosomal gel consisting of 1.5 % of Carbopol 940 was found to be superior to the gel formulations since the physical appearance shows good properties. The reading pH value at 6.39 ± 0.03 while the viscosity is at 3569.11 cps. The formulated gel showed good stability after the centrifugation test by not showing statistical differences in pH value, viscosity, and spreadability before and after the test.

The membrane filtration sterility testing shows the absence of microorganisms. Hence, from all the results it was shown that mangishtha extract was well incorporated into carbopol formulation to form herbosomal gel.

This developed herbosomal gel using extract has good pH value, viscosity, spreadability, and stability against varying temperatures and storage conditions. Besides, the anti-inflammatory activity of both gel formulation and crude extract also exhibited good and promising results. Hence, it can be concluded that the extract has the potential to be developed as a commercial anti-inflammatory gel for medical use with further preclinical and long-term stability study can be conducted.

Ethics and Consent: No animal studies were performed

Competing Interests: No competing interests

Grant Information: Non-funded by any agency

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