CHAPTER 3

"EXPERIMENTAL"

CHAPTER 3: EXPERIMENTAL

3.1 MATERIALS AND EQUIPMENT

3.1.1 Chemicals and excipients

Various chemicals used in the present work are enlisted in Table 3.1

Table 3.1: List of chemicals and excipients

Chemical	Manufacturing Company
Acetone	SD fine-chemie limited, Mumbai
Acetonitrile, HPLC	SD fine-chemie limited, Mumbai
Casein digest agar	Himedia laboratories pvt. Ltd., Mumbai
Cellulose acetate phthalate (CAP)	Central drug house, New Delhi
Dimethyl sulphoxide (DMSO)	SD fine-chemie limited, Mumbai
Disodium hydrogen phosphate	Loba chemie pvt. Ltd., Mumbai
DTNB [5,5′-dithiobis (2-nitrobenzoic acid)]	Loba chemie pvt. Ltd., Mumbai
EDTA(Ethylene diamine tetra acetic acid)	Loba chemie pvt. Ltd., Mumbai
ELISA kits for assay cytokines	Krishgen biosystem Ltd., Mumbai, India
Ethanol	Central drug house, New Delhi
Glacial acetic acid	Central drug house, New Delhi
Hexa decyl trimethyl ammonia	Sigma aldrich, Mumbai
Hydrochloric acid	Central drug house, New Delhi
Hydrogen peroxide	SD fine-chemie limited, Mumbai
Isooctane	Loba chemie pvt. Ltd., Mumbai
Lactobacillus acidophilus	Hi-tech bio sciences Pune, India
Saccharomyces boulardii	Hi-tech bio sciences Pune, India
Light liquid paraffin	Loba chemie pvt. Ltd., Mumbai

MCC (PH101)	Qualikems fine chemicals pvt. Ltd., New Delhi. India
Mesalamine	Loba chemie pvt. Ltd., Mumbai
Methanol	Himedia laboratories pvt. Ltd., Mumbai
Naphthylethylenediamine	Central drug house, New Delhi
n-Octanol	Himedia laboratories pvt. Ltd., Mumbai
O-dianisidine	Loba chemie pvt. Ltd., Mumbai
Pectin	Himedia laboratories pvt. Ltd., Mumbai
Potassium dihydrogen phosphate	Loba chemie pvt. Ltd., Mumbai
Sodium chloride	Loba chemie pvt. Ltd., Mumbai
Sodium nitroprusside	Central drug house, New Delhi
Span 80	Loba chemie pvt. Ltd., Mumbai
Sulfanilic acid reagent	Central drug house, New Delhi
Thiobarbituric acid	Loba chemie pvt. Ltd., Mumbai
Trichloroacetic acid	Himedia laboratories pvt. Ltd., Mumbai
Trinitrobenzenesulfonic acid	Loba Chemie pvt. Ltd., Mumbai

3.1.2 Equipment used

Table 3.2: List of different instruments used in present research

Instrument	**Model No**	**Manufacturer**
Autoclave	29861208	MULTITECH
Bath sonicator	40050	Steryl medi equip system, Chennai
Biosafety cabinet	1590VII-48-24-24	Klenzaios contamination pvt. Ltd
Digital melting point apparatus	DB- 31354	Decibels instruments, (perfit India)
Digital weighing balance	AB265-S/ FACT	Mettler toledo AG, Laboratory & weighing technology, Switzerland

DSC	SIIO 6300	SIIO, Japan
Fourier transform infrared spectroscopy (FTIR)	Thermo Nicolet -380	Thermo scientific, U.S.A.
High-performance liquid chromatography	515	Waters, USA
Hot air oven	PT 400	Perfit India
Melting point apparatus	31354	Decibels, India
Micropipette	(100-1000 µl)	Tarsons, India
Motic digital microscope	DMWBI-223ASC	Motic
Motic digital microscope	BA 310	Motic incorporation, Hong Kong
pH meter	Pico	Lab India instruments pvt ltd.
Plate reader	680	BIO-RAD, USA
Remi centrifuge	R-8C, FBLC-5910	Remi instruments division Maharastra, India
SEM	SUU8020	Hitachi
Shaking Incubator	LSB-1005RE	Daihan lab tech. co. ltd. Korea
Stability chamber	400G	Thermolab, India
Tissue homogenizer	WiseMix HG-15D	Dathan scientific, New Delhi, India
UV-VIS spectrophotometer	Lambda 25 (Parkin Elmer)	Shimadzu, Japan
Zeta sizer	DLS 4 C	Beckman coulter, USA
Vacuum pump	65	Welch, USA
Water purifier	Milli-Q	Millipore, USA
Weighing balance	AB265	Mettler toledo, UK
Magnetic mixer	122	Remi work, India

Table 3.3: List of various computer software used in the present research

Software	Manufacturer	Use
Graph Pad Instat 3	GraphPad Software Inc., USA	Statistical Analysis
MS-Office 2007	Microsoft Corp., USA	Thesis Writing

3.2 PREFORMULATION STUDIES OF MESALAMINE

Preformulation study is essential for determining the drug's physical and chemical properties before incorporating it in formulation development. (Gopinath *et al.*, 2011). The nature of the drug highly affects the processing parameters like the method of preparation, entrapment efficiency, compatibility and pharmacokinetics response of the formulation (Chaurasia *et al.*, 2016). Preformulation studies are an essential method of developing a dosage form that is safe, effective, and stable. Preformulation tests were carried out in order to provide the best conditions for a therapeutically useful delivery system, as shown in Figure.3.1.

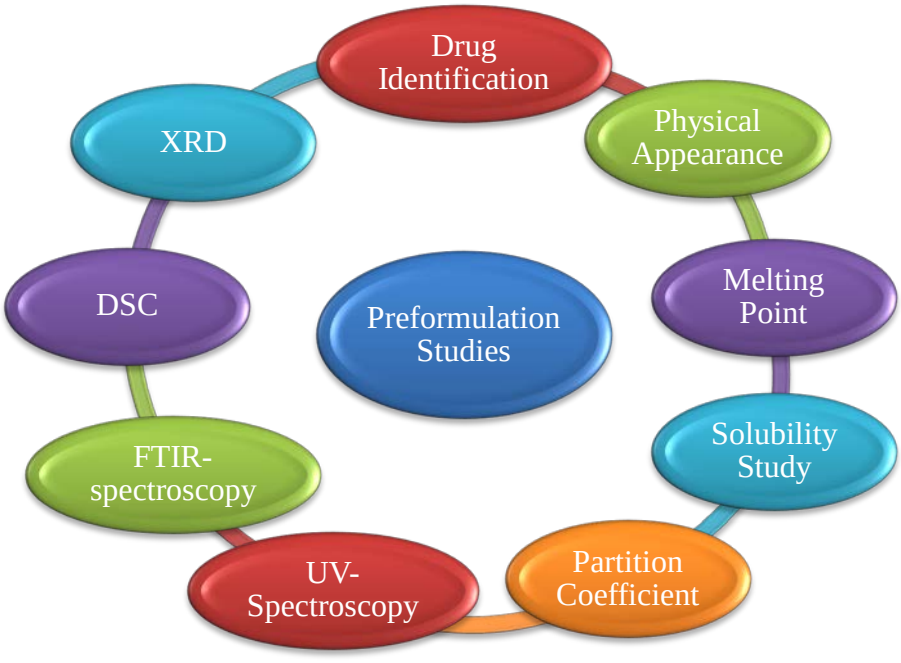

Figure 3.1: Preformulation study parameters of Mesalamine.

3.3 ORGANOLEPTIC PROPERTIES

3.3.1 Drug morphology

Mesalamine is amorphous.

3.3.2 Physical state

Mesalamine is a white powder.

3.3.3 Melting point

A compound's melting point is an important physical property. The melting point of a substance can be used to identify it and determine its purity. The melting point of the drug was determined using a capillary melting point device. To observe the melting point range, a small amount of the drug was filled in the previously one-sided sealed capillary and the melting point was analyzed in a melting point device.

3.3.4 Partition coefficient

A chemical compound's partition coefficient is a thermodynamic measure of its hydrophilicity-lipophilicity balance. The partition coefficient is the ratio of the unionized drug dispersed between the organic and aqueous phases (Figure.3.2).

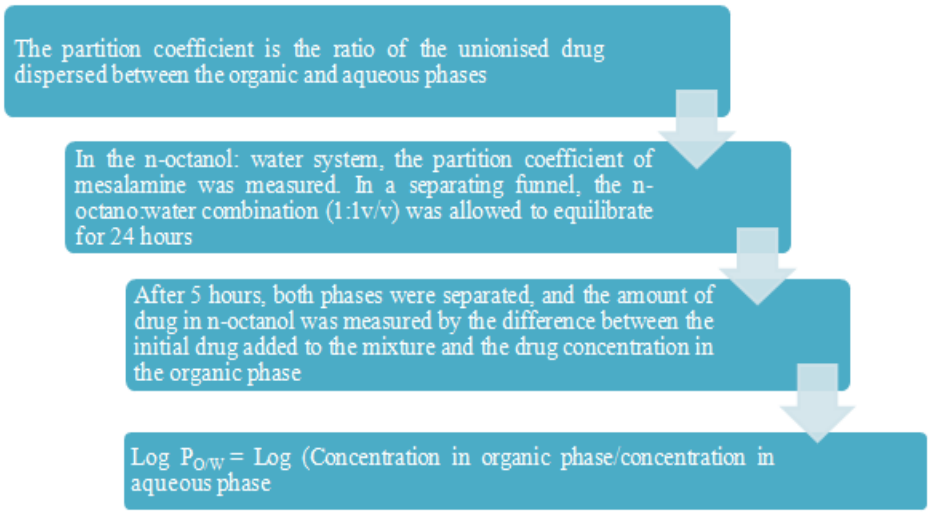

Figure 3.2: Flowchart for the partition coefficient evaluation.

3.3.5 Solubility study

It is the amount of solute dissolved in a given solvent under standard temperature, pressure, and pH conditions to produce a homogeneous solution. The drug's solubility was conducted in various solvents, including methanol, ethanol, chloroform, and DMSO. To perform these experiments, a small amount of solvent was chosen, and incremental amounts of drug were added to the solvent until the solution was utterly saturated with the drug. The solution was filtered after complete saturation and the concentration of the drug in the solution was calculated by measuring the UV absorbance of the solution and comparing it to the drug's standard curve.

3.3.6 Fourier transform infrared (FTIR) spectroscopy for compatibility study

IR study was performed for identification and structural analysis of the sample using FTIR spectrometry (Figure.3.3). FTIR transmission of pure Mesalamine was obtained using an FTIR spectrophotometer (Basu *et al.*, 2010).

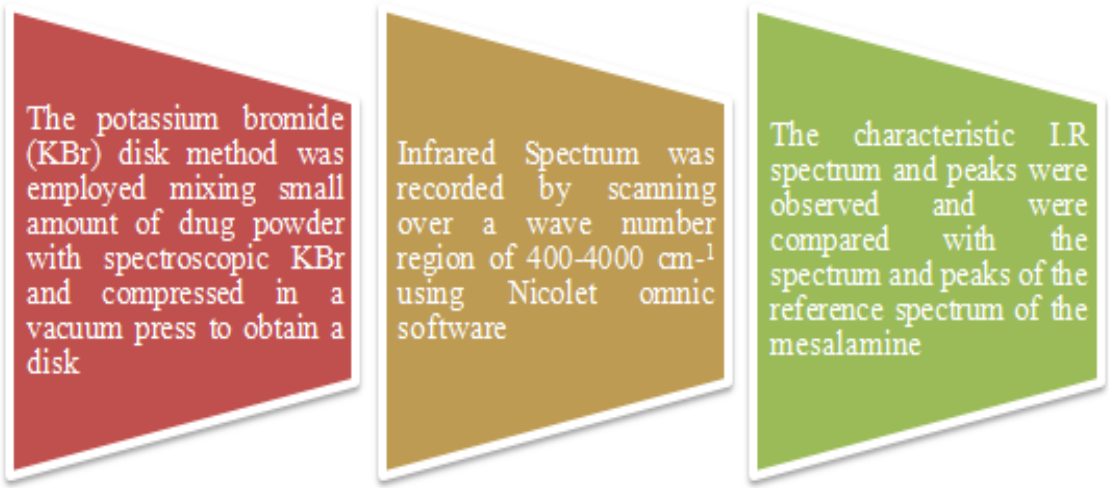

Figure 3.3: Procedure for performing FTIR Spectroscopy.

3.4 DEVELOPMENT OF THE ANALYTICAL METHODS

3.4.1 UV-visible spectroscopy studies

3.4.1.1 Preparation of standard curve of the drug in PBS 7.4

Mesalamine (10 mg) was accurately weighed and taken in a clean and dry 100 ml volumetric flask and the volume was made with PBS pH 7.4 up to 100 ml, to produce a stock solution of 100µg/ml (Figure.3.4).

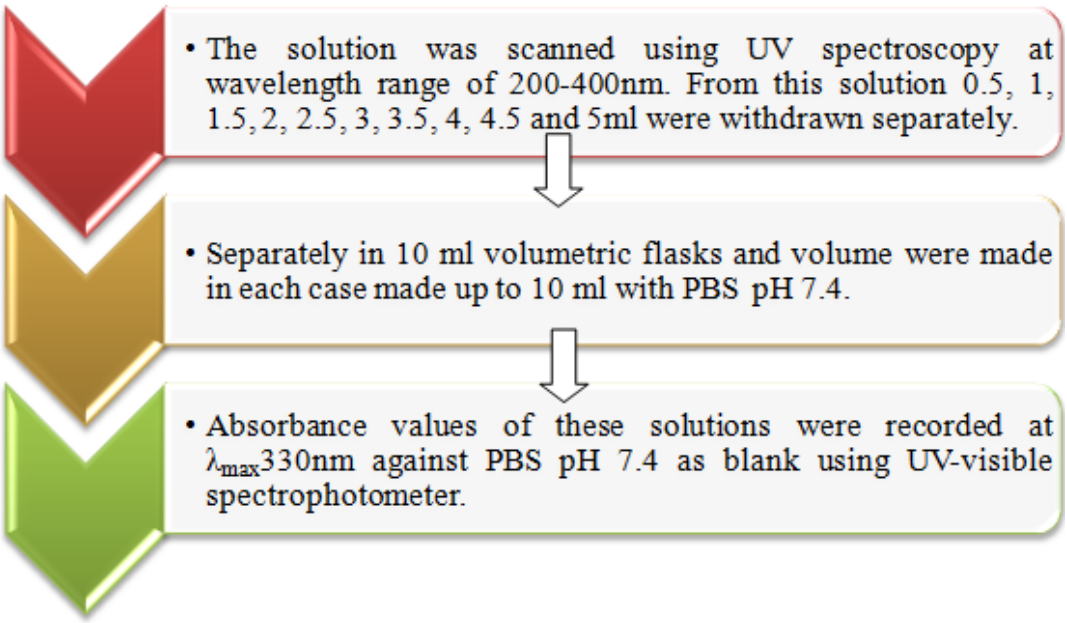

Figure 3.4: Schematic representation for plotting a standard curve of Mesalamine in PBS 7.4.

3.4.1.2 Preparation of standard curve of the drug in 0.1N HCl

Mesalamine (10 mg) was accurately weighed and taken in a clean and dry 100 ml volumetric flask and the volume was made with 0.1N HCl up to 100 ml to produce a stock solution of 100µg/ml. A standard curve was plotted between concentrations vs. absorbance. Statistical parameters related to calibration curves like slope, intercept, regression coefficient, standard deviation and relative standard deviation were determined (Figure.3.5).

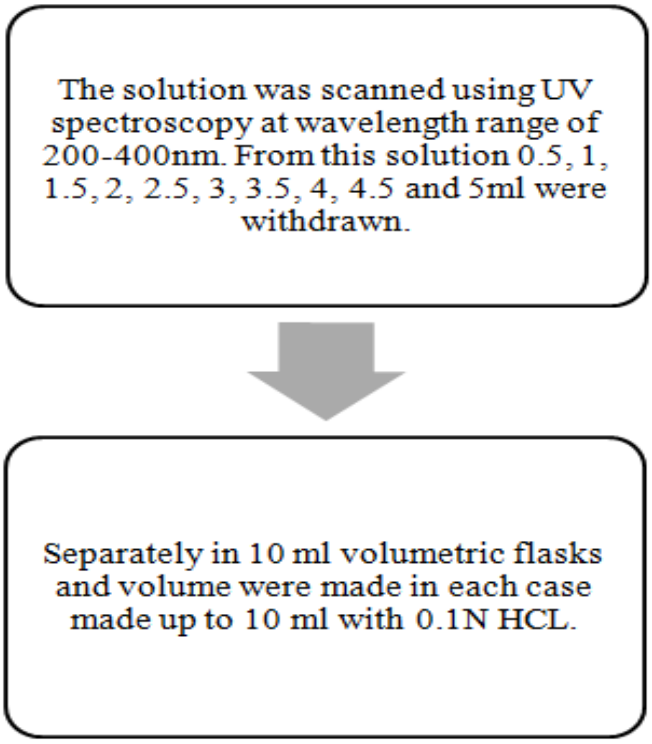

Figure 3.5: Schematic representation for plotting a standard curve of Mesalamine in 0.1N HCl.

3.5 HPLC METHOD FOR MESALAMINE DETERMINATION

Waters HPLC Chromatograph with UV detector and Empower software was utilized to carry out the different determinations. ODS column Phenomenex (250 x 4.6 mm) was used to perform separations at room temperature. A mixture of acetonitrile, tertbutyl-ammonium chloride (0.1M), and phosphate buffer of pH 6.2 was selected as the mobile phase in a ratio of 85:15:1 (v/v). Sonication, filtration and pumping of the mobile phase was done at a flow rate of 1.0 ml/min. The samples were injected through Rheodyne injection and the eluted components were detected by UV detector at 330 nm.

3.6 SCREENING OF PROBIOTIC STRAIN BASED ON THE ANTIOXIDANT AND INFLAMMATORY PROPERTY

3.6.1 Nitric Oxide assay for determination of the antioxidant potential of probiotics

By utilizing (Eom *et al.*, 2018) the formula, IC_{50} value has been calculated.

Nitric oxide scavenged (%) = [$A_{control}$ - A_{test}/ $A_{control}$] x 100

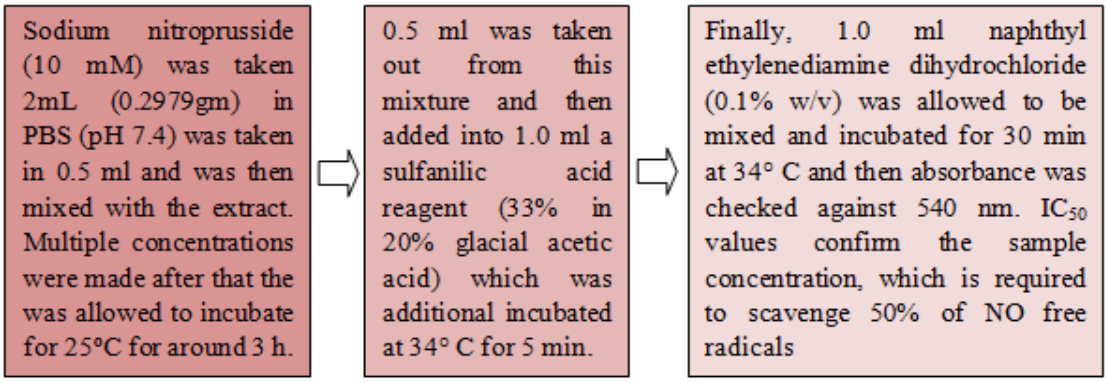

Figure 3.6: Procedure for performing nitric Oxide assay.

The IC_{50} value was achieved by plotting the different bacteria concentrations and scavenging activity, which is known as the total antioxidant potential required to decrease the primary NO radical concentration by 50% (Figure 3.6). Finally, the measurements were done. Where A control = Absorbance of control reaction and A test = Absorbance in the presence of the sample. The IC_{50} value was determined from the equation of the plotted graph of scavenging activity against the different concentrations of samples. The equation is- Y = mx+c, Where, y = % inhibition of NO activity (50), x = Concentration, c = constant

3.6.2 Cell culture

Culturing of Caco-2 cell lines of human colon carcinoma was done in a medium

consisting of RPMI 1640 50% (v/v), fetal bovine serum 15% (v/v), Dulbecco's modified Eagle's medium DMEM 35% (v/v), and 1% (w/v) penicillin-streptomycin. NIH3T3 cells were cultured with DMEM supplemented with 10% FBS and 1% (w/v) penicillin-streptomycin in 5% of CO_2 atmosphere conditions with 95% relative humidity and at 37 °C.

3.6.3 Inflamed intestinal barrier model for anti-inflammatory activity estimation of probiotics

For the cultivation of Caco-2 cells, 96 well-tissue plates were used. The cells were exposed to pro-inflammatory cytokines consisting of IL-1β and LPS at 50 and 1000 ng/ml for 24 hrs at 37 °C for inflammation induction. The untreated Caco-2 cells act as a control. To confirm an inflammation in the cell model, the centrifugation of extracellular media was done at 15,000 rpm for 5 minutes and IL-8 production was tested using sandwich enzyme-linked immunosorbent assay (ELISA) kits (Sigma-Aldrich Mumbai) as directed by the manufacturers (Van *et al.*, 2010).

3.7 FORMULATION DEVELOPMENT

In present studies, two types of microcarrier drug delivery systems were developed to successfully deliver mesalamine and *S. boulardii* in the colonic region. The first one is microparticles and the second is the pellets-based delivery system. Both microcarriers are further coated with enteric-coated CAP polymer.

3.7.1 Preparation and optimization of pectin microparticles

The pectin microparticles were prepared by dehydration technique (Jain *et al.*, 2009). Mesalamine was dissolved in DMSO, followed by probiotic *S. boulardii* (10^9 CFU/ml) in the PBS 7.4 solution, and then this suspended solution was added to the Mesalamine solution. A solution of pectin was prepared in another beaker and drug-probiotic dispersion was poured into it. Around 10 ml was dispersed from this dispersion into 50 ml of isooctane containing a span 80 (1.0% w/v) and continuously

stirred to achieve stable water/oil emulsion at various speeds. The dispersion was rapidly cooled to 15° C and about 50 ml of acetone was poured into it to dehydrate the pectin droplets. Microparticles were then continuously stirred for 30 minutes at 1000 rpm at a temperature of 30 °C to allow the solvent to be fully evaporated, followed by drying and then stored in a desiccator.

3.7.1.1 Coating of pectin microparticles

Pectin microparticle coating was done using the oil-in-oil solvent evaporation method (Lorenzo *et al.*, 1998). This method dispersed 50g pectin microparticles in 10 ml of organic solvent mixture (acetone: ethanol, 9:1) containing CAP. The prepared organic phase was added into 70ml of light liquid paraffin containing 1% (w/v) span 80, followed by constant stirring for 3 hrs at 1200 rpm at room temperature to evaporate the solvent. Finally, n-hexane has been used for the washing of coated microparticles. After this, microparticles are filtered, dried and stored in a desiccator.

3.7.2 Characterization of prepared microparticles

3.7.2.1 Drug loading and entrapment efficiency

The centrifugation of microparticle dispersion was done at 12000 rpm for 30 min. The supernatant was collected in a test tube (Zafar *et al.*, 2021). The microparticles were washed 3 times using water, centrifuged and supernatants were collected. The amount per drug in the supernatant was determined using the calibration curve. The following formula estimated these parameters.

$$\% \text{ Entrapment efficiency} = \frac{\text{Total drug} - \text{Free drug}}{\text{Total drug}} \times 100$$

$$\% \text{ Drug loading} = \frac{W_{\text{Total}} \times W_{\text{Free}}}{W_{\text{Polymers}}} \times 100$$

W_{total}, W_{free}, and $W_{polymers}$ are the weight of the drug added in the system, analyzed weight of the drug in supernatant and weight of polymers added in the system, correspondingly.

3.7.2.2 Particle morphology

Scanning electron microscopy was used to evaluate the prepared uncoated and coated microparticles' shape and surface morphology. This procedure was completed by sprinkling a dried powdered microparticle sample on double adhesive tape fixed on an aluminum stub. Coating of stubs was done with gold to a thickness of ~300 Å and SEM obtained the images of samples at 1300x magnification (JSM-840; Joel, Tokyo, Japan) (Singh *et al.*, 2022).

3.7.2.3 Particle size analysis, polydispersity index and Zeta potential analysis.

Zeta sizer (Nano NS, Malvern, PA) was used to estimate the particle size of microparticles at pH (1.2 and 7.4). The polydispersity index of the optimized microparticles was determined by dynamic light scattering. For the estimation of Zeta potential, the same instrument was used (Khurana *et al.*, 2020).

3.7.2.4 FTIR Spectroscopy

FTIR study was performed for the identification and structural analysis of samples. FTIR transmittance of uncoated and coated microparticles was obtained using an FTIR spectrophotometer (Agilent). The potassium bromide (KBr) disk method was used by adding a small amount of sample powder with spectroscopic KBr and compressing it in a vacuum press to obtain a disk. The infrared Spectrum was recorded by scanning over a wave number region of 400-4000 cm^{-1} using empowers software (Basu *et al.*, 2010).

3.7.2.5 X-ray diffraction (XRD) determination

XRD analysis was conducted for optimized formulations of uncoated and coated microparticles. Measurements of X-ray scattering angle were performed with a copper anode fixed to the diffractometer (45 kV, 40 mA) in a wide-angle X-ray diffractometer (D8 Advance, BRUKER, Germany) with 2θ Angle (Caddeo *et al.*, 2014).

3.7.2.6 Differential scanning calorimetry (DSC)

DSC verifies the drug nature and thermal transitions, polymorphic transitions engaged in energy variation through the formulation process. The DSC curve of Mesalamine, pectin, MCC and formulation were assessed by DSC (Mettler Toledo stare DS822, Germany) in perforated aluminum-sealed pans at a heating rate of 5°C/min as of 10 to 340°C with Nitrogen gas (50ml/s) (Negi *et al.*, 2020).

3.7.2.7 *In vitro* drug release

In vitro release study of prepared coated microparticles (Mesalamine and probiotic) was carried out using USP dissolution apparatus (Dissolution test apparatus, Lab India DS 8000 instrument) type I in the 900 ml medium at 37°C at a speed of 100 rpm. The aqueous dispersion of mesalamine and probiotic microparticles was prepared immediately before merging into a dialysis bag of 12–14 KDa MWCO and beginning the dissolution study. Three types of dissolution media were used 1^{st} - SGF having (pH 1.2), 2^{nd} -SIF (pH 6.8), and 3^{rd} - SCF (pH 7.4) with and without 2% rat cecal content was utilized to simulate the entire G.I.T transit environment. The release study was performed in simulated gastric fluid (pH 1.2) for 2 hrs. Additionally, it was completed in simulated intestinal fluid (pH 6.8) for 3 hrs, followed by SCF (pH 7.4) for 19 hrs. At various intervals, the samples were collected, diluted, and examined by UV spectrophotometer at 334 nm. After the sample collection, 5 ml of fresh media was placed in the dissolution media to maintain the volume (Kang *et al.*, 2020).

3.7.2.8 Release and viability count of *S. boulardii* from the coated microparticles during dissolution.

The Release and *S. boulardii* viability were verified along with coated and uncoated Mesalamine-probiotic microparticles for 24 hrs. To avoid misconception, this procedure was carried out three times. *S. boulardii* colonies were counter and resulted in CFU/g of yeast.(Avachat *et al.*, 2016).

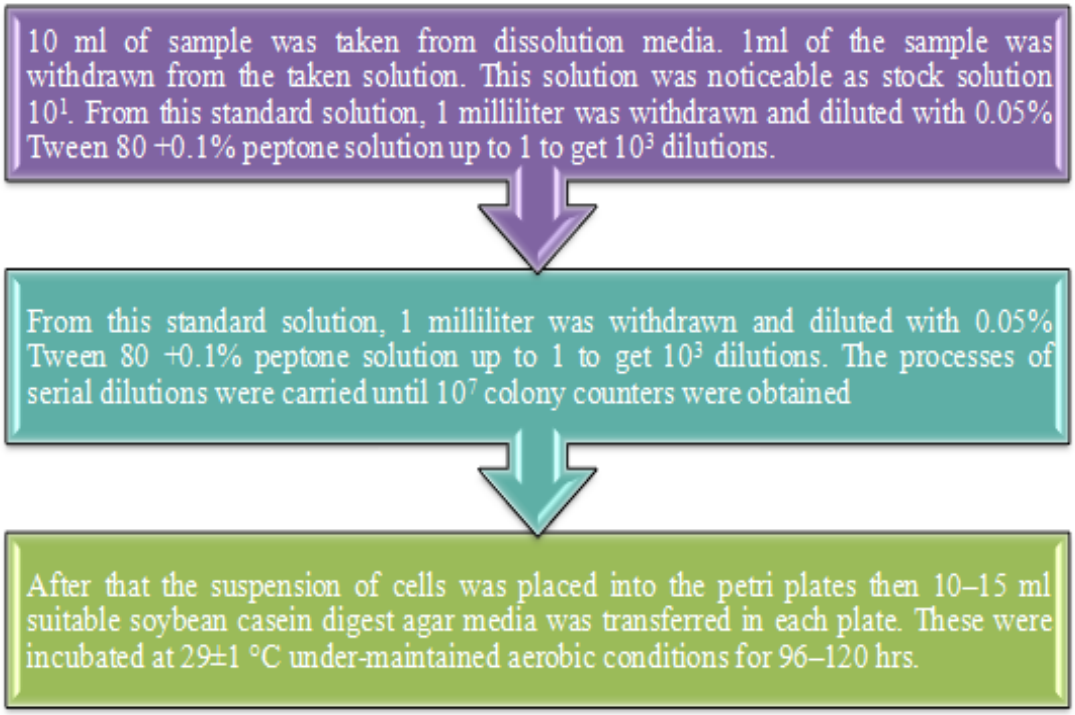

Figure 3.7: Procedure for Release and viability count of *S. boulardii* .

3.8 *IN VIVO* EFFICACY STUDIES USING TNBS-INDUCED COLITIS MODEL IN WISTAR RAT

3.8.1 *In vivo* studies

The study was carried out on 36 either-sex Wistar rats weighing 180-260 g. Animals were obtained from ISFCP, Moga, and Punjab, India. Animals were kept at ambient temperature (21±10°C) and relative humidity (55 ± 5%) with a fixed 12 hrs light/dark cycle. The experimental layout was approved as ISFCP/IAEC/CPCSEA/Meeting No 26/2020/Protocol no.434 by the Institutional Animal Ethical Committee (IAEC) as per the committee's guidelines for control and supervision of experiments on animals (CPCSEA).

TABLE 3.4: Different animal groups used *in vivo* studies

S. No.	Groups	No. of Animals
1	Normal control (No disease and no treatment)	6
2	Disease control	6
3	Colitis + placebo group (Inert material formulation), oral route	6
4	Colitis + mesalamine microparticles (23 mg/kg, oral route)	6
5	Colitis + probiotic microparticles (10^9 CFU), oral route	6
6	Colitis + coated microparticles of Mesalamine (23 mg/kg) + probiotic (10^9 CFU) for 15 days, by oral route	6

The animal division was carried out in six groups. Group I served as normal control and Group II served as disease control. The oral route treated Group III with colitis + placebo group (inert material formulation). Group IV received colitis + mesalamine microparticles (23 mg/kg) via the oral route. The oral route treated Group V with colitis + probiotic (10^9 CFU) microparticles. Group VI was treated with colitis + coated microparticles formulation of mesalamine (23mg/kg) + probiotic 10^9 CFU via Oral route. Fifteen days of oral treatment were given using oral gavage (Table 3.4).

3.8.2 Induction of colitis by TNBS

Colitis was induced with slight modifications. Fasting of rats was done for 48 hrs with free access to water and then anesthetized with Ketamine (50 mg/kg). A rubber cannula was rectally inserted into the colon with 8 cm proximal to the anus. TNBS (2, 4, 6-Trinitrobenzenesulfonic acid) (10 mg dissolved in 0.25 ml of 50% ethanol) was administered into the colon lumen using a rubber probe (total volume 0.5 ml solution). The disease control group received 0.5 ml 50% ethanol (v/v) (Morris *et al.*, 1989). The animal division was carried out in six groups. Group I served as normal control and Group II served as disease control. The oral route treated Group III with

colitis + placebo group (Inert material formulation). Group IV received colitis + Mesalamine microparticles (23 mg/kg) via the oral route. The oral route treated Group V with colitis + probiotic (10^9 CFU) microparticles. Group VI was treated with colitis + coated microparticles formulation of [Mesalamine (23mg/kg) + probiotic (10^9 CFU)] via Oral route. Fifteen days of oral treatment were given using oral gavage, as shown in Figure 3.8**.**

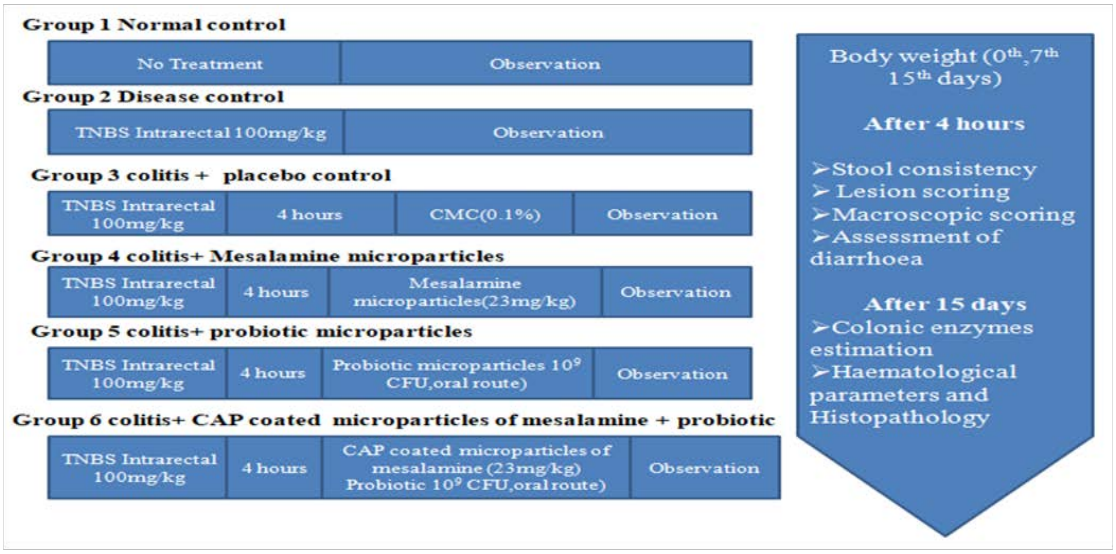

Figure 3.8 Treatment schedule after the induction of colitis.

3.8.3 Morphological studies

3.8.3.1 Macroscopic characters assessment

The macroscopic colon observation is a significant part of UC severity monitoring. The severity of colitis was measured by an observer unfamiliar with the treatment schedule (Nidhi *et al.*, 2017). The extent of colon inflammation has been visually assessed, and the treatment's effectiveness can be directly judged. The scores are given in Table 3.5 and Figure 3.9.

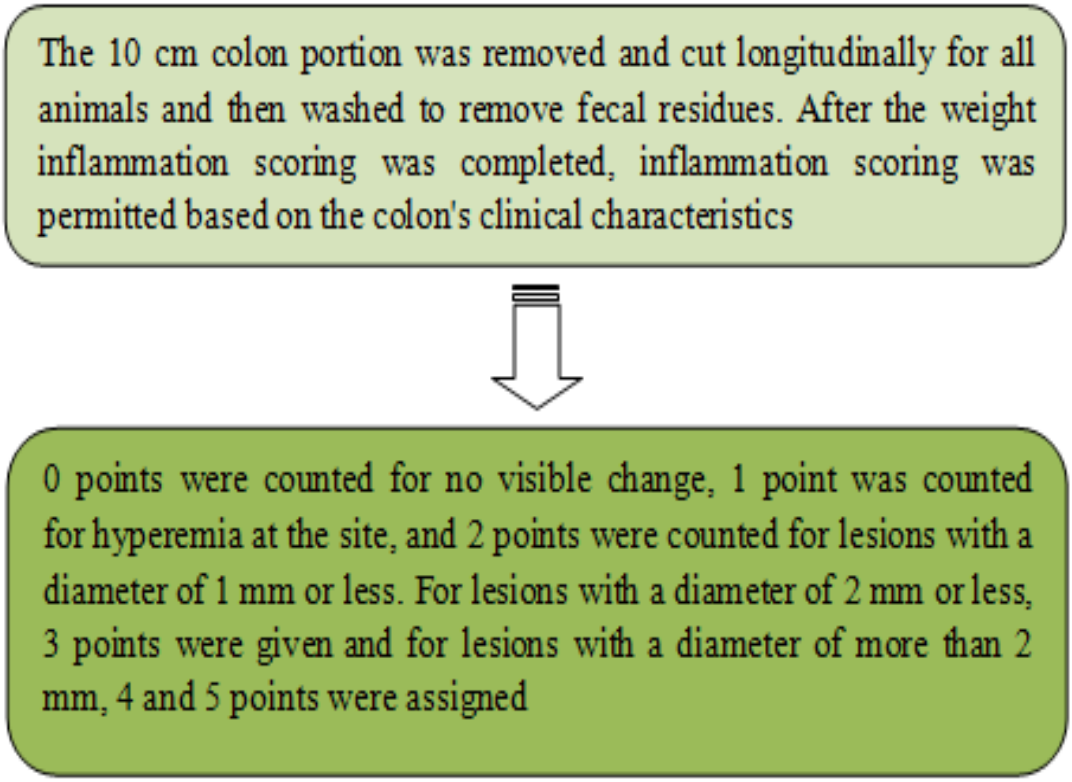

Figure: 3.9: Procedure for macroscopic character assessment.

3.8.3.2 Body weight evaluation

Body weights of rats were evaluated before sacrificing animals at the beginning of the 0^{th} day before induction and after colitis induction after (0^{th} day), 7^{th} day and final day (15^{th} day). (Ahmad *et al.*, 2021). Scores are given in Table 3.5.

3.8.3.3 Diarrhoea assessment

Wistar rats were housed in a cage with a clean white sheet after colitis induction by TNBS and were assessed for 4 hrs for the occurrence of diarrhea, fecal discharge, fecal consistency and fecal bleeding (Ferri *et al.*, 2019). From each group, fecal material was analyzed regularly, and the recordings were ranked accordingly for diarrhea evaluation.

Table 3.5: Scores for change in weight, consistency of stool and lesions scores

For weight change	Stool Consistency	Lesion Score
0% -0 score	Well-formed pellets-0 Score	No lesion-0 Score
1- 5 % -1 score	Hard-1 Score	Hyperemia at sites-1 Score
5- 10 % - 2 Score	Semisolid-2 Score	Scoreless than 1 mm-2 diameter
10 to 20 % - 3 Score	Pasty-3 Score	Lesion having a diameter less than 2 mm-3 Score
Above 20 %-4 Score	Liquid-4 Score	Lesion having a diameter less than 3 mm-4 Score

3.8.4 Biochemical studies

A portion of colonic tissue (remaining from the histopathological study) samples (n=6) were homogenized in 10% (w/v) of ice-cold potassium phosphate buffer (pH 7.4) using Elvenjan homogenizer (Remi Motors Ltd., Mumbai) and the homogenate was used for the measurement of myeloperoxidase activity (MPO), lipid peroxidation and reduced glutathione (GSH) (Wang *et al.*, 2016).

3.8.4.1 Determination of colonic MPO activity

One unit of MPO activity is defined as the change in absorbance per min by 1.0 at room temperature in the reaction (Figure 3.10). It has been calculated by using the following formula.

MPO activity = X/weight of the piece of tissue taken

Where X=10×change in absorbance per min/volume of supernatant taken in the final concentration.

3.8.4.2 Determination of colonic lipid peroxides concentration (LPO)

The above procedure was repeated for the blank solution without homogenate (Figure 3.11). The amount of lipid peroxide was determined by using the following formula:

(3 * OD of the sample)/ (0.156 * mg pr) = n moles of MDA/mg pr

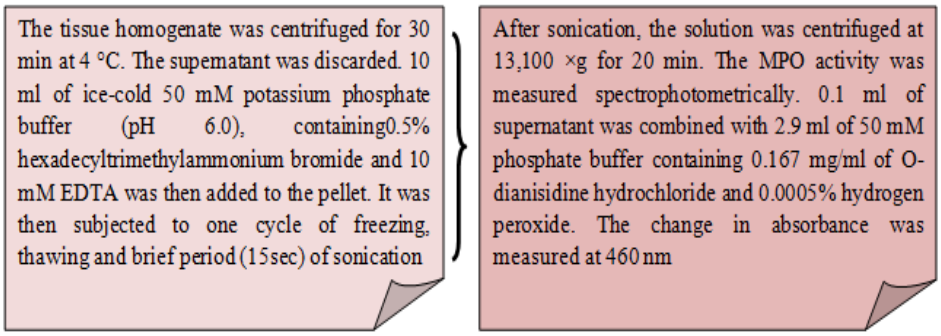

Figure 3.10: Process for determination of colonic MPO activity.

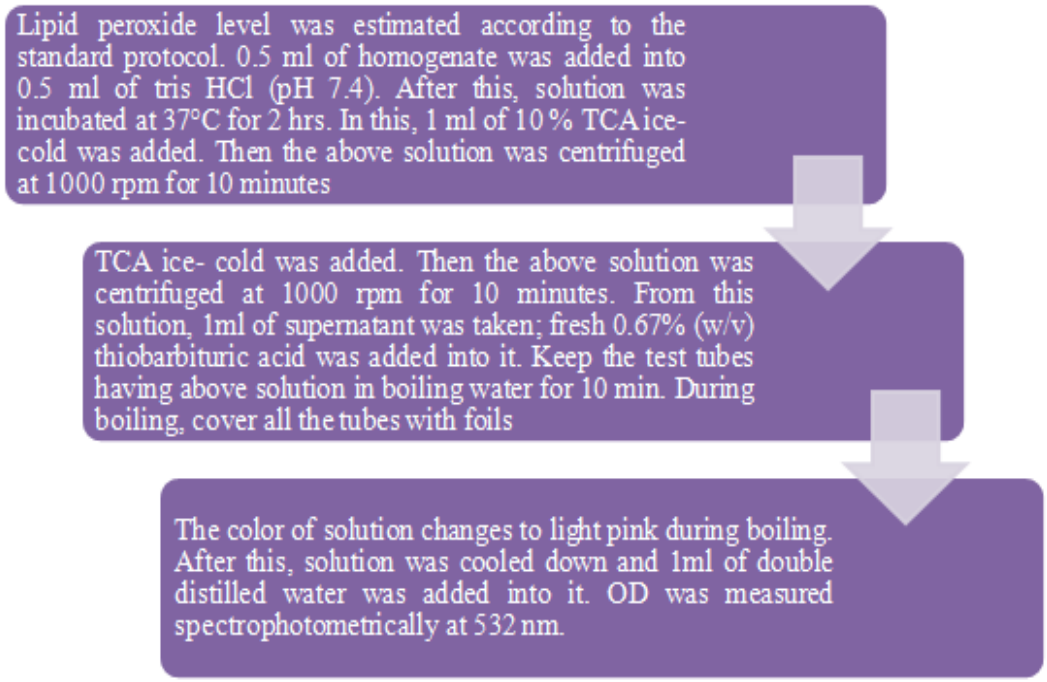

Figure 3.11: Process for determination of colonic lipid peroxides concentration (LPO).

3.8.4.3 Determination of colonic GSH contents

The assay is based on forming a relatively stable yellow product when sulphydryl groups react with 5, 5-dithio-bis-2-nitro-benzoic acid (DTNB). Briefly, proteins were precipitated using 10% TCA, centrifuged and 0.5 ml of the supernatant was mixed with 0.2 M phosphate buffer (pH 8.0) and 10 mM DTNB. This mixture was incubated

for 10 min and the absorbance was measured at 412 nm against the appropriate blank. The results were expressed as µmol/g of wet tissue weight.

3.8.5 Measurement of Serum-Markers

3.8.5.1 Determination of C-reactive protein

For withdrawing blood from each animal, retro-orbital puncturing was used. A latex agglutination method was used to conduct the C-reactive protein assay. CRP Turbilatex test kit was used for the same (Abdin *et al.*, 2008).

3.8.5.2 Determination of WBC count and ESR

The total leukocyte white blood cell count was analyzed using diluted blood samples and the solution of Turk (3% acetic acid) was used at a ratio of 1:200 for dilution. Counting was done using a hemocytometer (Viana *et al.*, 2012; Cioffi *et al.*, 2015). A collection of 0.8 ml for the ESR measurement in Westergren's method was used. From each group, around 0.8 ml of blood sample collection was obtained in tubes containing EDTA (Ethylenediaminetetraacetic acid) and the ESR level was assessed.

3.9 HISTOPATHOLOGY STUDY

The colon (2 cm) of each rat (n=6) was taken and fixed with 10% formalin, followed by cutting into 5 µm thickness, staining with hematoxylin-eosin dye and finally, the interpretation was evaluated. These colon sections were analyzed for inflammatory changes such as tissue damage, cell infiltration, nucleus damage, and necrotic focal points (Morsy *et al.*, 2019).

3.10 STABILITY STUDY

Stability studies of the optimized coated microparticle formulations were conceded according to the guidelines of ICH. The optimized drug- probiotic-loaded microparticles, were conducted at $25^{o}C \pm 2^{o}$ C and $40^{o}C \pm 2^{o}$ C, 60% RH ± 5%and 75 ± 5% RH, respectively. In the coated microparticles, the formulation was sealed in

aluminum foils. Furthermore, significant changes were observed for 6 months. Finally, the estimation of change in percentage of drug entrapment, moisture content and cumulative percentage of drug and probiotic release was evaluated (Kumar *et al.*, 2020).

3.11 PHARMACOKINETIC STUDIES FOR MICROPARTICLES FORMULATION

Pharmacokinetic studies followed the procedure reported by (Wei H *et al.*, 1989) with slight variation. The uncoated and coated Mesalamine-probiotic microparticles were administered to rats through the oral route [Wistar rats (either sex), 190-260 g] via a polyethylene cannula (diameter: 2mm) with 1 ml of (1% carboxymethyl cellulose solution), at a dose correspondent to 23mg/kg. Blood samples (1.0 ml) were collected from retro orbitalis and placed into heparinized tubes at 0.5,1,2,4,6,8,10,12,14,16 and 24 hrs after administration. The supernatants were obtained through this procedure, redissolved in 0.1ml of the mobile phase, vortexes for 3 min and centrifuged at 10,000 rpm for 3 min. Supernatants (0.02 ml) were injected for HPLC analysis of mesalamine under the conditions explained in the next section. Further pharmacokinetic parameters were determined by plotting a plasma concentration-time graph, and C_{max} and T_{max} were calculated directly. AUC was determined using the trapezoidal method, followed by the calculation of MRT. The observations were expressed in terms of mean and standard deviation. Statistical treatment was employed to test the determined parameters using a T-test. Also, the elimination rate constant and elimination half-life were calculated from the plasma concentration-time curve using standard formulas.

3.12 STATISTICAL EVALUATION

Data were expressed as means ± SD. For statistical evaluations, one-way ANOVA and Tukey–Kramer post-test was applied. A value of p-value <0.05 was considered significant.

3.13 PREPARATION AND OPTIMIZATION OF PELLETS FORMULATION

3.13.1 Preparation of uncoated pellets

Mesalamine and *S. boulardii* loaded pellets were formulated by the extrusion–spheronization method (Figure 3.12). Pellets were formulated from 5 gms of blend powder consisting of 1.0% Mesalamine, 10^9 CFU of *S. boulardii*, 29.0 % of pectin, and 70.0% of MCC. These components were mixed with distilled water and converted into appropriate dough mass for pelletization. Eleven non-identical formulations MP1 to MP11 were formulated by changing concentrations of bulking agent (MCC), pectin as core agent up to a definite limit to obtain the desired pellets size. MP10 formulation was the final optimized pellet formulation among the ten prepared formulations, which was further coated with enteric-coated polymer CAP. At 40°C, the pellets were dried using an oven (Figure 3.13). The optimization of the quantity of pectin and MCC was done. The choice of the most excellent formulation was based on pellet size, percentage yield, Angle of repose, entrapment efficiency and friability. The yield was calculated as a percentage of the weight of dried pellets obtained divided by the initial weight of solid mass before extrusion- spheronization (Kaur *et al.*, 2020) by utilizing the following equation :

$$\text{Batch yield (\%)} = \frac{\text{Amount of pellets obtained}}{\text{The total amount of drug,polymer and excipient}} \times 100$$

Figure 3.12: Extrusion spheronizer for preparation of pellets.

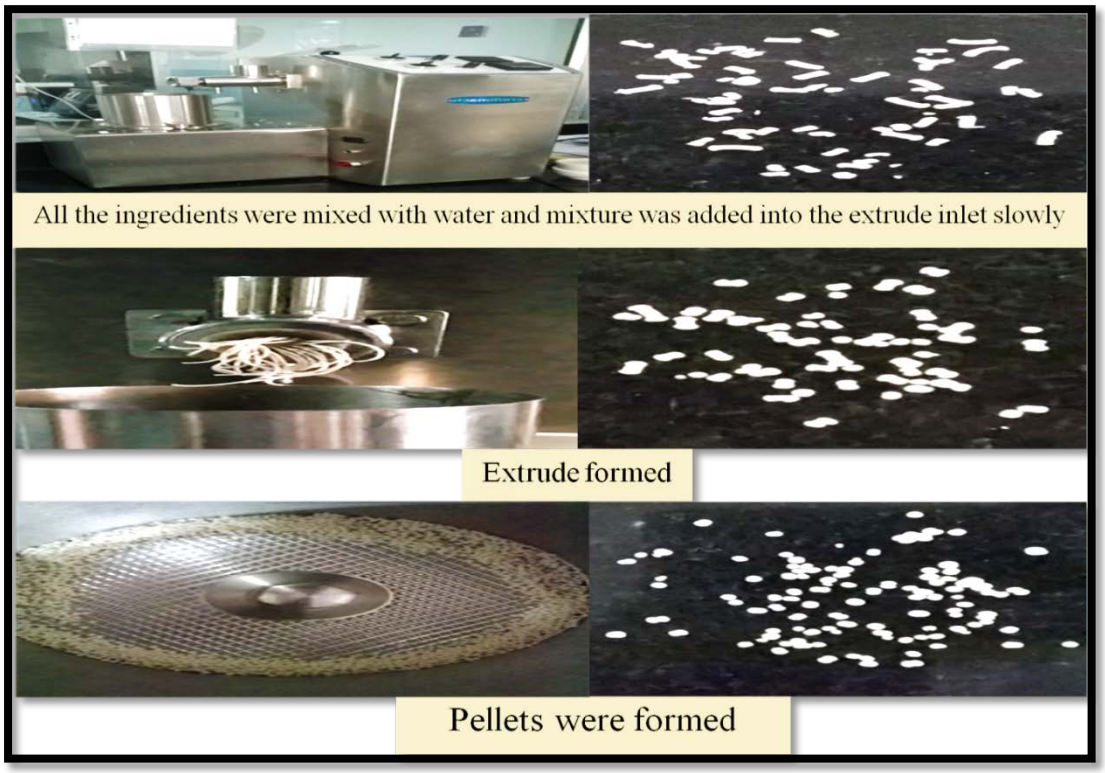

Figure 3.13: Spheronization and extrusion process.

3.13.2 Coated pellets preparation

CAP was employed for drug-probiotic-loaded pellets coating. Correctly weighed CAP was obtained to prepare four different concentrations i.e., 2.5%, 5%, 7.5% and 10 % to get optimized coating concentration (Table 3.6). CAP was dissolved in the organic solvent mixture (acetone: ethanol, 9:1) and then a peristaltic pump pipe was rinsed using an organic solvent. The coating solution was filled in the tube with maximum assurance that no air bubbles were in the pipe. Additionally, the pipe was joined with accela cota spray gun (Figure 3.14). The coating pan window was shut and process parameters like flow rate, air pressure, drum speed and temperature were adjusted accordingly. The coating was done in intervals, like spraying atomized coating solution on pellets for 1 min. They were then dried in hot air for 1 min. Likewise, repeat this 3 times (Pandey *et al.*, 2018).

Table 3.6: Optimization of coating solution

S. No.	**Concentration (%)**	**Flow rate (ml/min)**	**Drum speed** (rpm)	**Temperature** (ºC)	**Air pressure** (kg/cm²)
1.	2.5	1	20	50	5
2.	5	1	20	50	10
3.	7	1	20	50	10
4.	10	1	20	50	10

Figure 3.14: Accela –cota coating instrument.

3.14 *IN VITRO* CHARACTERIZATION OF DRUG-PROBIOTIC-LOADED PELLETS

3.14.1 Drug loading and entrapment efficiency

The centrifugation of microparticle dispersion was done at 12000 rpm for 30 min. The supernatant was collected in a test tube. The microparticles were washed 3 times by using water, centrifuged and supernatants were collected. The amount per drug in the supernatant was determined using the calibration curve. The following formula estimated these parameters.

$$\%\text{Entrapment efficiency} = \frac{\text{Total drug} - \text{Free drug}}{\text{Total drug}} \times 100$$

$$\%\text{Drug loading} = \frac{W_{\text{Total}} \times W_{\text{Free}}}{W_{\text{Polymers}}} \times 100$$

W_{total}, W_{free}, and $W_{polymers}$ are the weight of drug added in the system, analyzed weight of the drug in the supernatant and the weight of polymers added in the system, correspondingly.

3.14.2 Pellets morphology

With the help of motic microscope, pellets size estimation was done. A 10x objective lens was used to determine pellet size. A dirt-free glass slide and glycerin about a drop were laid on the slide. The pellet was placed above the glass slide and the size of the pellets was determined.

3.14.3 Sphericity Studies

The shape and the area of the pellets were investigated by optical microscopic image analysis. Pellet size and shape were measured using an optical DMW2-223 digital microscope (Motic Instruments) equipped with a 1/3" CCD camera imaging accessory and computer-controlled image analysis software (Motic Images 2000, 1.3 version). 50 pellets from each batch were selected and analyzed by microscopic image analysis

technique and various parameters like aspect ratio, roundness and circularity were used to assess the shape of the pellets. The particle size data was further processed into the aspect ratio, circularity factor and roundness using the following equations:

$$\text{Aspect ratio} = \frac{D_{max.}}{D_{min.}}$$

$$\text{Circularity factor} = \frac{4\pi A}{P^2}$$

$$\text{Roundness} = \frac{P^2}{12.56 \times A}$$

3.14.4 Micrometric properties

Pellets were characterized for flow properties such as Angle of repose, bulk density, tapped density, Hausner's ratio, Carr's and compressibility index and flow behavior was studied**(**Vanitha *et al.*,2018**;** Reji *et al.*,2022) The results were expressed as mean values of three determinations.

3.14.4.1 Angle of repose

The Angle of repose of pellets determined the flow properties. Briefly, sample is allowed to fall gently through a funnel onto a hard surface from a height of 2.5cm (Table 3.7). The height of the pile and diameter were noted. The angle of repose was determined by using the following formula.

$$\text{Angle of repose } (\theta) = \tan^{-1} (h/r)$$

Where h is the height and r is the radius of the powder cone.

Table 3.7: The empirical relation between the flow properties and the repose angle.

Flow properties	Angle of repose (◦)
Excellent	<25
Good	25-30
Passable	30-40
Very poor	>40

3.14.4.2 Bulk density

Pellets were accurately weighed 10 gm and were gently poured through a glass funnel into calibrated 100 ml measuring cylinder. The surface was made smooth carefully with an application of pressure. The volume occupied by the sample was recorded and bulk density (g/ml) was calculated and recorded in the table using the following formula.

$$\text{Bulk density} = \frac{\text{Weight of sample taken}}{\text{Bulk volume}}$$

3.14.4.3 Tapped density

Like the bulk density, tapped density was observed by tapping the cylinder 100 times from a 3-inch height using Electrotab tapped density apparatus after pouring the pellets into the measuring cylinder and recording the tapped volume. Finally, the tapped density was recorded by using the formula.

$$\text{Tapped density} = \frac{\text{Weight of sample taken}}{\text{Tapped volume}}$$

3.14.4.4 Hausner's ratio

Hausner's ratio is a number that is correlated to the flow ability of pellets (Table 3.8). The following formula calculated it:

$$\text{Hausner's ratio} = \frac{\rho t}{\rho b}$$

Table 3.8: Flow property determination based on Hausner's ratio.

Flow properties	Hausner's ratio
Excellent	1.00-1.11
Good	1.12-1.18
Fair	1.19-1.25
Passable	1.26-1.34
Poor	1.35-1.45
Very poor	1.46-1.59
Very, very poor	>1.60

3.14.4.5 Carr's index

Carr's index is an indication of the compressibility of pellets (Table 3.9).

The following formula calculated it:

$$\text{Carr's index (\%)} = \frac{\rho t - \rho b}{\rho t} \times 100$$

Table 3.9: Flow property determination based on Carr's index.

Flow properties	Carr's index
Excellent	≤10
Good	11-15
Fair	16-20
Passable	21-25
Poor	26-31
Very poor	32-37
Very, very poor	>38

3.14.4.6 Friability

Accurately weighed 10 gm of pellets were placed on a sieve with a 0.85 mm aperture with 25 glass beads of 3 mm diameter and then both were placed in Roche's friabilator (Veego Scientific, India) for 100 revolutions at 25 rpm speed. The pellets were collected and placed on the sieve with a 0.85 mm aperture. The smaller particles were allowed to pass through the sieve and pellets were reweighed. The friability was determined as the percentage loss of mass of pellets after the test was recorded.

$$\text{Friability} = \frac{\text{Initial weight} - \text{final weight}}{\text{Initial weight}}$$

3.14.5 Determination of moisture content

Karl Fischer titration was conducted to determine the moisture contents of the pellets formulation.

3.14.6 Mucoadhesive test

The mucoadhesive efficiency of non-coated pellets was evaluated on the colon mucosa of Wistar rat. A longitudinal cut was made in the colon with a varying length and purified by (pH 7.4) phosphate buffer. Further, the colon mucosa was placed on dissection tray support, spread and placed by the pins. Uncoated pellets were placed on the mucosa of the colon were placed in a container at a specific relative humidity (60±10%) and room temperature for 20 minutes. After 20 minutes, using phosphate buffer, the mucosa was washed for 5 minutes at the flow rate of 1 ml/min with the help of a syringe. At last, the total number of pellets adhered to the mucosa was counted. For better mucoadhesion, maximum pellets should have adhered with the mucosa (Desai *et al.*, 2020).

3.14.7 Spectral characterization through FTIR

FTIR study was performed for the identification and structural analysis of samples. FTIR transmittance of uncoated and coated pellets was obtained using an FTIR spectrophotometer (Agilent). The potassium bromide (KBr) disk method was used by adding a small amount of sample powder with spectroscopic KBr and compressing it in a vacuum press to obtain a disk. The infrared Spectrum was recorded by scanning over a wavenumber region of 400-4000 cm^{-1} using empower software (Sharma *et al.*, 2013).

3.14.8 X-ray powder diffraction (XRD)

XRD analyses were executed for optimized uncoated and coated pellets formulations. Determination of the X-ray scattering angle was evaluated with a copper anode fixed to the diffractometer (45 kV, 40 mA) in a wide-angle X-ray diffractometer (D8 Advance, BRUKER, Germany) with 2θ Angle (Varshosaz *et al.*,2011).

3.14.9 Differential scanning calorimetry (DSC)

DSC verifies the drug nature and thermal transitions, polymorphic transitions engaged

in energy variation through the formulation process. The DSC curve of Mesalamine, pectin, MCC and formulation were assessed by DSC (Mettler Toledo stare DS822, Germany) in perforated aluminum-sealed pans at a heating rate of 5°C/min as of 10 to 340°C with nitrogen gas (50 ml/s) (Yadav *et al.*,2021).

3.14.10 *In vitro* drug release

The *in vitro* drug release studies of uncoated and coated pellets were conducted using a USP XXIII dissolution test apparatus I (Dissolution Test apparatus, Lab India DS 8000 instruments) in the 900 ml medium at 37°C at a speed of 100 rpm. Confirmed pellets corresponding to 50 mg of mesalamine were weighed and placed in the dissolution basket medium. The dissolution tests were done for 2 hrs in SGF (pH 1.2, 900 ml) and 3 hrs in the SIF (pH 6.8, 900 ml) and approximately 19 hrs in SCF (pH 7.4, 900 ml) (Total 24 hrs). At various intervals, the samples were collected, diluted, and examined by UV spectrophotometer method at 334 nm. After the sample collection, 5 ml of fresh media was placed in the dissolution media to maintain the volume.

3.14.11 Release and viability count of *S. boulardii* from the pellets during dissolution.

The release and *S. boulardii* viability were verified with mesalamine, a formulated coated pellet for 24 hrs. 10 ml of sample was taken from dissolution media. 1 ml of the sample was withdrawn from the taken solution. This solution was noticeable as stock solution 10^{-1}. From this standard solution, 1 ml was withdrawn and diluted with 0.05% Tween 80 + 0.1% peptone solution up to 1 to get 10^{-3} dilutions. The serial dilutions were carried out until 10^{-7} colony counters were obtained. After that, the suspension of cells was placed into the petri plates and 10–15 ml of soybean casein digest agar media was transferred to each plate. These were incubated at 29±1°C under-maintained aerobic conditions for 96–120 hrs. To avoid misconception, this

procedure was carried out three times. The colonies of *S. boulardii* were counted and the result was expressed as CFU/g of yeast (Graff *et al.*, 2008).

3.15 *IN VIVO* STUDIES

3.15.1 Induction of colitis and drug administration in Wistar rats

Wistar rats were randomly divided into six groups, each having six rats. Colitis was induced according to the procedure described by Morris *et al* 1989 with slight modification. The rats were randomly divided into different treatment groups and fasted for 48 hrs with free water availability. After that, using Ketamine (50 mg/kg), rats were anesthetized. A rubber cannula was rectally inserted into the colon site by 8 cm proximal to the anus. TNBS (10 mg dissolved in 0.25 ml of 50% ethanol) was given into the colon's lumen by the rubber probe (0.5 ml solution total volume). Before eliminating the rubber probe, air (2 ml) was inserted to expand the TNBS solution totally in the colon. The rats were examined for watery stools and rectal bleeding. For 2 days, the rats were housed without any treatment schedule, so maximum colitis development could occur at the colon site. Group I served as Normal control and Group II served as disease control; Group III was treated with TNBS +Placebo group (Inert pellets) oral route. Group IV received TNBS + plain Mesalamine pellets (23 mg/kg, oral route), Group V was treated with TNBS + plain probiotic (10^9 CFU) pellets, Group VI was treated with TNBS + coated pellets containing mesalamine 23 mg/kg and probiotic (10^9 CFU), oral route. All these treatments were given for 15 days.

3.15.2 Macroscopic characters assessment

The colitis severity was determined by a person unknown to the treatment schedule. The 10 cm colon part was separated for all animals, longitudinally cut, and then washed to remove fecal residues. After that, scoring of inflammation was done based on the clinical characteristics of the colon. 0 points were counted for no visible change, 1 point for hyperemia at sites, and 2 points for lesions with a diameter l mm

or less. Lesions with a diameter of 2 mm or less as 3 points and lesions with a diameter of more than 2 mm are counted as 4 and 5 points correspondingly**,** scores (Ge *et al.,* 2022). The macroscopic inspection of the colon was an imperative feature for severity monitoring of UC. The degree of inflammation of the colon was evaluated visually and the treatment efficiency was directly judged (Motavallian *et al.*, 2012).

Percentage change in weight = (Weight on the first day – Weight on last day) / weight on the first day *100

3.15.2.1 Assessment of body weight

Body weights of Wistar rats were confirmed on the initial 0^{th} day before the colitis induction and after colitis induction (on the 7^{th}day), 10^{th} day, and (15^{th} day) last day before animal sacrificing (Valcheva *et al.*, 2018).

3.15.2.2 Diarrhoea assessment

After TNBS colitis induction, Wistar rats were accommodated in a cage with a white background and were confirmed for approximately 4 hrs for the occurrence of diarrhea, fecal discharge quantity, fecal consistency and fecal bleeding. In each group's treatment schedule, fecal content was observed daily to assess diarrhea; the recordings were ranked accordingly (Direito *et al.*, 2019).

3.16 DETERMINATION OF MPO, LPO AND GSH

The activity measurement of MPO, LPO and GSH was performed to quantify the colitis severity. The colon tissue (600 mg) was weighed, added instantly to a test tube and stored at 4° C. The tissue was obtained from the storage, 5.5 ml of saline was put in, the mixture was homogenized, and tissue homogenate was centrifuged at 3000 rpm for 15 min at 4°C. The supernatant was collected to measure MPO, LPO and GSH activity (Kannan *et al.*, 2013).

3.17 MEASUREMENT OF SERUM-MARKERS

3.17.1 Determination of C-reactive protein

For withdrawing blood from each animal, retro-orbital puncturing was used. A latex agglutination method was used to conduct the C-reactive protein assay. CRPA Turbilatex test kit was used for the same (Xavier *et al.*, 2006).

3.17.2 Determination of WBC count and ESR

The total leukocyte white blood cell (WBC) count was analyzed using diluted blood samples and the solution of Turk (3% acetic acid) was used at a ratio of 1:200 for dilution. Counting was done using a hemocytometer (Viana *et al.*,2012). A collection of 0.8 ml of for the ESR measurement in Westergren's method was used. From each group, around 0.8 ml of blood sample collection was obtained in tubes containing EDTA (Ethylenediaminetetraacetic acid) and the ESR level was assessed (Morsy *et al.*, 2019).

3.18 HISTOPATHOLOGICAL ASSESSMENT

A distal colon 2 cm part from every rat (n=6) was collected and was fixed in 10% phosphate-buffered formalin and fixed in paraffin afterward, which was furtherly cut into 5 μm thickness and stained with hematoxylin-eosin. These colon sections were inspected for epithelial damage, necrotic foci, architectural changes, cell infiltration, and ulceration (Mandlik *et al.*, 2021).

3.19 STABILITY STUDY

Stability studies of the optimized pellets formulations were conceded according to the guidelines of ICH. The optimized formulation (MP11), drug-probiotic loaded pellets were conducted at $25^{o}C \pm 2^{o}$ C and $40^{o}C \pm 2^{o}$ C,60% RH ± 5% and 75 ± 5% RH, respectively. The coated pellets formulation was sealed in aluminum foils. Furthermore, significant changes were observed for 6 months. Finally, the estimation of change in percent drug entrapment, moisture content and percentage cumulative

drug release was evaluated (Kumar *et al.*, 2020).

3.20 PHARMACOKINETIC STUDIES OF PREPARED PELLETS FORMULATION

The uncoated and coated Mesalamine-probiotic pellets were administered to rats through the oral route.(Gulbake *et al.*, 2016) [Wistar rats (either sex), 190-260 g] via a polyethylene cannula (diameter: 2 mm) with 1ml of (1% carboxymethyl cellulose solution) at a dose corresponding to 23 mg/kg. Blood samples (1.0 ml) were collected from retro orbitalis and placed in heparinized tubes at 0.5,1,2,4,6,8,10,12,14,16 and 24 hrs after administration. The supernatants were obtained through this procedure, redissolved in 0.1ml of the mobile phase, vortexed for 3 min and centrifuged at 10,000 rpm for 3 min. Supernatants (0.02 ml) were injected for HPLC analysis of Mesalamine under the conditions explained in the next section. Further pharmacokinetic parameters were determined by plotting a plasma concentration-time graph, and C_{max} and T_{max} were calculated directly. AUC was determined using the trapezoidal method, followed by the calculation of MRT. The observations were expressed in terms of mean, standard deviation. Statistical treatment was employed to test the determined parameters using a T-test. Also, the elimination rate constant and elimination half-life were calculated from the plasma concentration-time curve using standard formulas.

3.21 STATISTICAL ANALYSES

Mean standard deviations were determined for all the results. Analysis of variance (ANOVA) was employed to compare the treatment and control groups' results. Graph pad Prism 5 software was used for all statistical treatments and graph plotting. The p-value < 0.05 was selected as a considerable limit.