CHAPTER 4

"RESULTS AND DISCUSSION"

CHAPTER 4: RESULTS AND DISCUSSION

4.1 PREFORMULATION PARAMETERS

Preformulation studies were performed to check the compatibility of the drug and excipients for the formulation preparation. Various preformulation parameters were performed, such as solubility study, melting point, FTIR study, UV analysis and partition coefficient. Results of the preformulation study suggested that Mesalamine was pure and free from impurities.

4.1.1 Physical state

Mesalamine was visually observed and found to be white powder and Organolaptic character.

4.1.2 Melting point

The melting point determination was performed to check the purity of the drug. The melting point of Mesalamine was found to be between 279-282 ºC (Table 4.1), which complies with the standard, *i.e.,* 283-285 ºC.

Table 4.1: Melting point of Mesalamine

Standard	Observed
283-285 ºC	279-282 ºC

4.1.3 Partition coefficient

The partition coefficient is the concentration ratio of a compound in a mixture of two immiscible phases at equilibrium. These coefficients measure the compound's solubility difference in these two phases (Table 4.2). The concentration of Mesalamine in both phases was estimated and partition coefficient was calculated using the formula partition coefficient = concentration in the organic phase (n-octanol)/ concentration in the aqueous phase (water).

Table 4.2: Partition coefficient of Mesalamine

Reported Log P	Observed Log P
1.1	0.98

4.1.4 Solubility profile of Mesalamine

Mesalamine was studied by preparing a saturated drug solution in these solvents. A saturated solution was prepared by adding the excess drug to these solvents in a screw-capped vial and kept in an orbital shaker for 24 hrs. The vials are centrifuged at 500 rpm. The amount of free drug in the supernatant was analyzed by spectrophotometer at 330 nm. The solubility of mesalamine in different solvents has been shown in Table 4.3.

Table 4.3: Solubility profile of Mesalamine in different solvents

S. No.	Solvent	Saturation solubility(mg/mL)	Interpretation
1	Water	1.21	Slightly Soluble
2	Methanol	0.65	Insoluble
3	0.1 N HCL	10.23	Soluble
4	PBS 7.4	10.13	Soluble
5	DMSO	30	Soluble

Table 4.4: Reported and observed IR peak of Mesalamine

Interpretation	Observed peak (cm^{-1})	Standard peak (cm^{-1})
C-N (stretch)	1348	1000-1400
N-H (stretch)	1559	1500-1650
COOC (stretch)	3254	2400-3400
OH (stretch)	3356	3200-3400

4.1.5 FTIR spectroscopy

FTIR spectra investigated the compatibility between the drug and polymer. The position of the characteristic peak in FTIR spectra of pure mesalamine was compared with the standard (Table 4.4). It was observed that there was no disappearance or shift in the bond position of functional groups, which interfered there is no interaction between the drug and excipient. FTIR spectrum of standard mesalamine is shown in Figure 4.1.

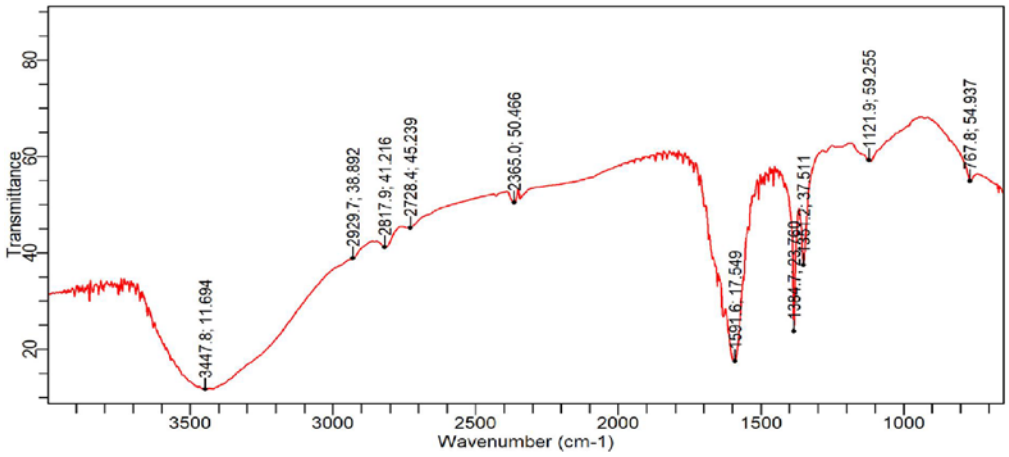
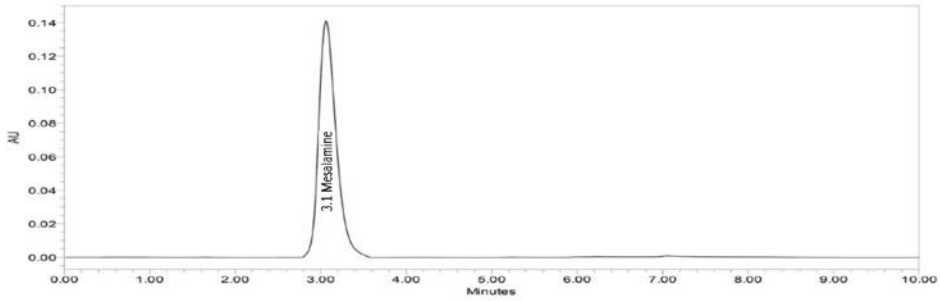


Figure 4.1: FTIR spectrum of Mesalamine.

4.2 ANALYTICAL METHOD DEVELOPMENT

An analytical method was developed and the retention time of Mesalamine was found to be 3.1 (Figure 4.2).



	Peak Name	RT	Area	% Area	Height
1	Sample_01	6.432	27283691	100.00	787862

Figure: 4.2 The chromatogram of Mesalamine.

4.2.1 Standard Curve of Mesalamine in PBS 7.4

Standard curve of Mesalamine in PBS 7.4 as shown in Table 4.5 and Figure 4.3.

Table 4.5 Calibration curve of Mesalamine in PBS 7.4

Concentration (µg/ml)	Absorbance
5	0.188
10	0.253
15	0.326
20	0.395
25	0.471
30	0.562

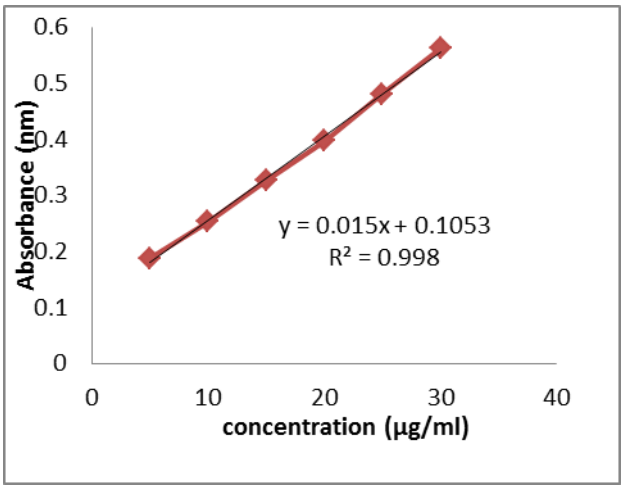


Figure 4.3: Standard curve of Mesalamine in PBS 7.4.

4.2.2 Standard curve of Mesalamine in 0.1 N HCL

Standard curve of Mesalamine in 0.1 N HCL as shown in table 4.6 and figure 4.4.

Table 4.6: Calibration curve of Mesalamine in 0.1N HCL

Concentration (µg/ml)	Absorbance
5	0.167
10	0.238
15	0.312
20	0.372
25	0.427
30	0.479

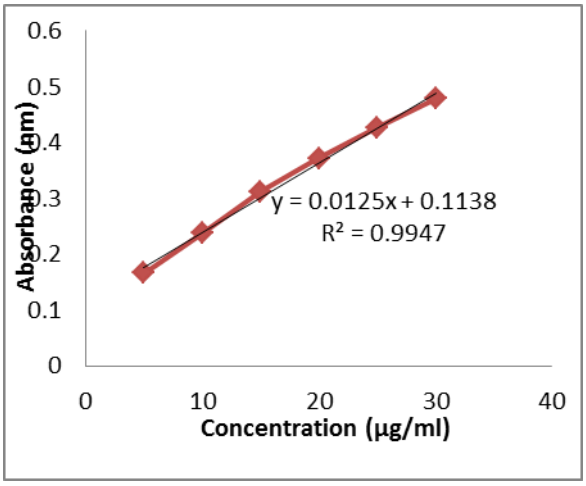


Figure 4.4: Standard curve of Mesalamine in 0.1 N HCL.

4.3 DETERMINATION OF ANTIOXIDANT ACTIVITY BY USING NITRIC OXIDE ASSAY

Antioxidant activity can be defined as a limitation or inhibition of nutrient oxidation by restraining oxidative chain reactions. Reactive oxygen species, including superoxides anions radicals, hydroxyl radicals and hydrogen peroxide, are the highly active oxygen free radicals. The antioxidant activity of probiotics may be used to delay or prevent oxidation.

Nitric oxide assay was performed to identify the antioxidant activity of *S.boulardii* and *L. acidophilus.* The results were conveyed as the dose necessary to cause 50% inhibitions by probiotic (IC_{50}). The IC_{50} value of *S.boulardii* and *L. acidophilus* was 58.06 µg/ml and 71.11 µg/ml, respectively. IC_{50} value between 50 µg/ml to 100 µg/ml is considered to ensure excellent intermediate antioxidant activity. The nitric oxide scavenging ability of *S.boulardii* was significantly higher than *L. acidophilus.* as shown in Table 4.7 and Figures 4.5 and 4.6. The result shows that *S.boulardii*. exhibit admirable nitric oxide scavenging activity, which is satisfactory for further use in the formulation.

Table: 4.7 Concentration and percentage inhibition of Nitric oxide activity of *S. boulardii* and *L. acidophilus*

Concentration (µg/ml)	**% inhibition of NO activity (*S. boulardii*)**	**% inhibition of NO activity (*L. acidophilus*)**
20	34.78±1.78	30.28±1.25
40	39.23±1.34	34.41±0.89
60	51.94±1.09	42.46±1.08
80	57.36±1.89	51.39±1.62
100	71.27±1.29	68.58±1.13

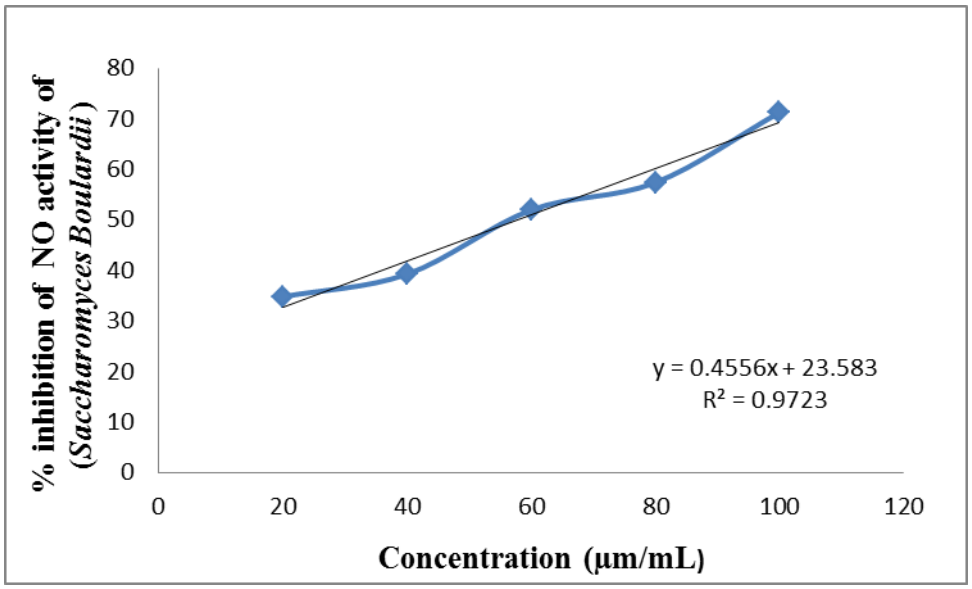


Figure 4.5: Percentage inhibition of NO activity of *S. boulardii* v/s concentration plot.

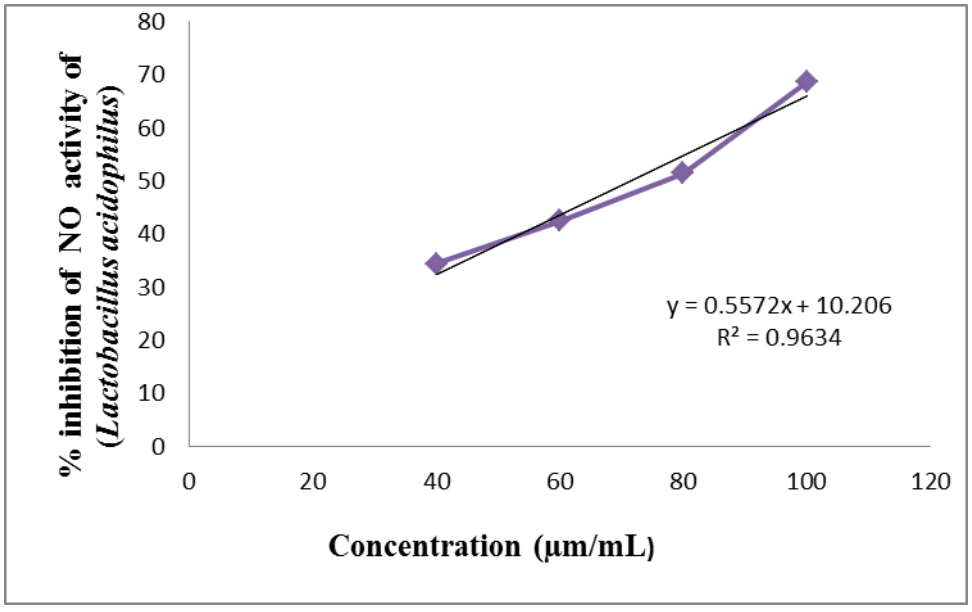


Figure 4.6: Percentage inhibition of NO activity of *L. acidophilus* v/s concentration plot.

4.3.1 Anti-inflammatory effect evaluation of probiotics (*S. boulardii* and *L. acidophilus*)

For the anti-inflammatory effect evaluation of both probiotics (*S. boulardii* and *L. acidophilus*). The Caco-2 model of inflammatory cells was used. The inflamed Caco-2 cells treated with pro-inflammatory mediators contain significant amounts of IL-8. The concentration of IL-8 was decreased from 2591±167 pg/ml to 513± 76 pg/ml and 663± 49 pg/ml after stimulation, verifying the anti-inflammation properties of both probiotics. From the observed results, it has been concluded that *S. boulardii* has a better anti-inflammatory effect (513± 76 pg/ml) than *L. acidophilus* (663± 49 pg/ml). *S. boulardii* was used further in the formulation due to its better antioxidant and anti-inflammatory activity.

4.4 PREPARATION, OPTIMIZATION AND CHARACTERIZATION OF MESALAMINE LOADED PROBIOTIC BASED MICROPARTICLES

4.4.1 Preparation & characterization of Mesalamine loaded probiotic based microparticles

The pectin-based Mesalamine and *S.boulardii*-loaded microparticles were prepared by dehydration and coated by an oil-in-oil solvent evaporation method. The microparticles were optimized using the different drug-to-polymer ratios, emulsifier concentration, stirring speed and stirring time.

4.4.2 Optimization of Mesalamine loaded probiotic based microparticles

The mesalamine-loaded probiotic-based microparticles were optimized using various parameters. The observed result confirmed that the formulation has good stability and meets the standards, as shown in Tables 4.8, 4.9, 4.10 and 4.11.

4.4.3 Drug loading and entrapment efficiency

The average drug loading capacity for mesalamine-coated microparticles was found to be 68.47±1.2%. The percentage average drug entrapment of mesalamine in coated microparticles was found to be 84.49% ± 3.5.

Table: 4.8 Optimization of drug-to-polymer ratio

Drug-to-polymer ratio	**Production yield(%)**	**Drug loading(%)**	**Entrapment efficiency(%)**	**Mean particle size(µm)**	***In vitro* drug release within 8 hrs (%)**
1:3	**98.70±1.65**	**20.69±1.34**	**82.76±0.45**	**9.14±0.96**	**86.21±1.19**
1:4	97.13±0.67	16.66±1.76	83.34±1.74	11.73±0.56	83.42±1.45
1:5	96.61±1.43	14.27±0.87	85.62±1.34	14.81±1.34	78.15±2.18
1:6	97.31±2.12	12.31±1.23	86.23±1.89	17.322±0.56	71.56±1.67

Table: 4.9 Optimization of emulsifier concentration

Emulsifier concentration Span 80(%)	**Production yield(%)**	**Drug loading(%)**	**Entrapment efficiency(%)**	**Mean particle size (µm)**	***In vitro* drug release within 8 hrs (%)**
0.5	93.08±2.43	17.82±0.71	71.28±2.56	27.67±0.56	74.82±2.45
1.0	96.19±0.78	19.32±1.26	77.23±1.89	25.35±0.39	70.31±0.67
1.5	95.67±0.56	20.34±1.56	81.38±2.47	19.38±0.92	75.36±0.82
2.0	**97.65±1.34**	**21.45±0.91**	**85.56±1.23**	**10.24±1.35**	**84.19±0.65**

Table: 4.10 Optimization of stirring speed

Stirring speed(rpm)	**Production yield(%)**	**Drug loading(%)**	**Entrapment efficiency(%)**	**Mean particle size (µm)**	***In vitro* drug release within 8 hrs (%)**
500	98.61±1.82	21.56±1.57	86.24±1.56	17.34±1.17	62.36±1.37
1000	95.23±2.31	22.80±0.37	91.22±2.21	12.32±1.45	73.67±2.82
1500	93.31±1.67	24.07±0.67	96.28±1.45	11.82±0.56	77.82±0.94
2000	**91.61±1.28**	**24.33±0.85**	**97.32±1.89**	**9.92±0.86**	**87.36±1.18**

Table: 4.11 Optimization of stirring time

Stirring time (min)	**Production yield(%)**	**Drug loading(%)**	**Entrapment efficiency(%)**	**Mean particle size (µm)**	***In vitro* drug release within 8 hrs (%)**
15	91.39±1.64	17.47±0.67	69.88±0.86	25.82±0.47	67.35±1.31
30	90.23±1.73	20.65±0.34	81.45±0.52	18.73±0.57	76.82±2.57
45	94.86±2.45	21.74±0.23	86.98±0.92	16.24±0.48	83.19±1.63
60	**94.25±0.82**	**22.71±0.53**	**90.84±1.32**	**10.28±0.53**	**97.16±0.94**

4.4.4 Particle morphology

Scanning electron microscopy of prepared uncoated and coated micro-particles was performed. Due to the presence of pectin, uncoated microparticles were slightly spherical with a slightly porous character (Figure 4.7). An almost complete spherical shape without porous visibility with a smooth surface has been evaluated in CAP coated microparticles. The uncoated and coated micro-particles were between 8-15 µm in size. For efficient drug delivery in the colonic site, microparticles with sizes varying from 5-15 µm are quite suitable.

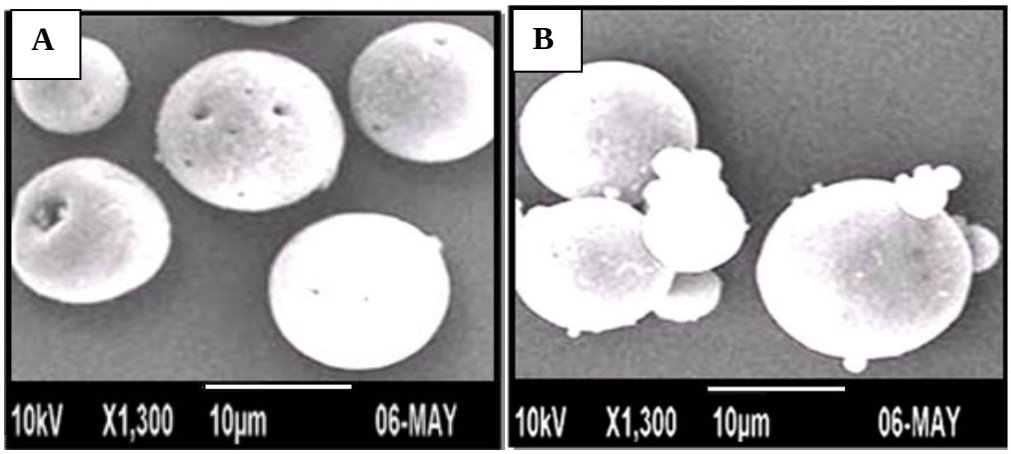


Figure 4.7: SEM images of uncoated (A) and coated (B) microparticles.

4.4.5 Determination of particle size, PDI and Zeta potential analysis

As per the literature survey, it has been concluded that the microparticle size ranges from 8-15 µm and can reach the affected colitis site. The mean particle size and size distribution were calculated in the Zeta sizer. The particle size was 9.14±0.96 and 11.61±0.5 µm for uncoated and coated microparticles. The polydispersity index of microparticles coated and uncoated was 0.245 and 0.267, respectively. It concluded a homogeneous state of the microparticles and an equal distribution of the same particle size. The Zeta potential of uncoated and coated microparticles was -26.78 ± 4.66 mV and -29.36 ± 3.36 mV, respectively, which ensures the uncoated and coated microparticle formulations were stable.

4.4.6 Compatibility study through FTIR spectroscopy

The compatibility between drugs and excipients was assessed through infrared spectroscopy. Various vibrational frequencies were recorded for uncoated and CAP-coated pectin-based Mesalamine microparticles. The specific IR peaks for Mesalamine originated at wave number (cm^{-1}) at 3447.8, 1591.6, 2929.7, 1121.9 and 1384.7 for –OH, -NH, -CH, -CO and C-C stretch, respectively, in uncoated microparticles containing pectin and Mesalamine as depicted in Figure 4.8 (A). A few characteristic bending peaks were also observed at 1121.9 and 767.8, corresponding

to in-plane and out-of-plane bending. Similarly, the vibrational frequencies were recorded for coated microparticles containing pectin, CAP and mesalamine Figure 4.9 (B). These findings revealed that all the significant peaks corresponding to Mesalamine were in the IR spectra of uncoated and coated microparticles. It indicated that mesalamine is fully compatible with polymers without any marked interference. The results also justified the stability of mesalamine in both the formulation without any indication of degradation.

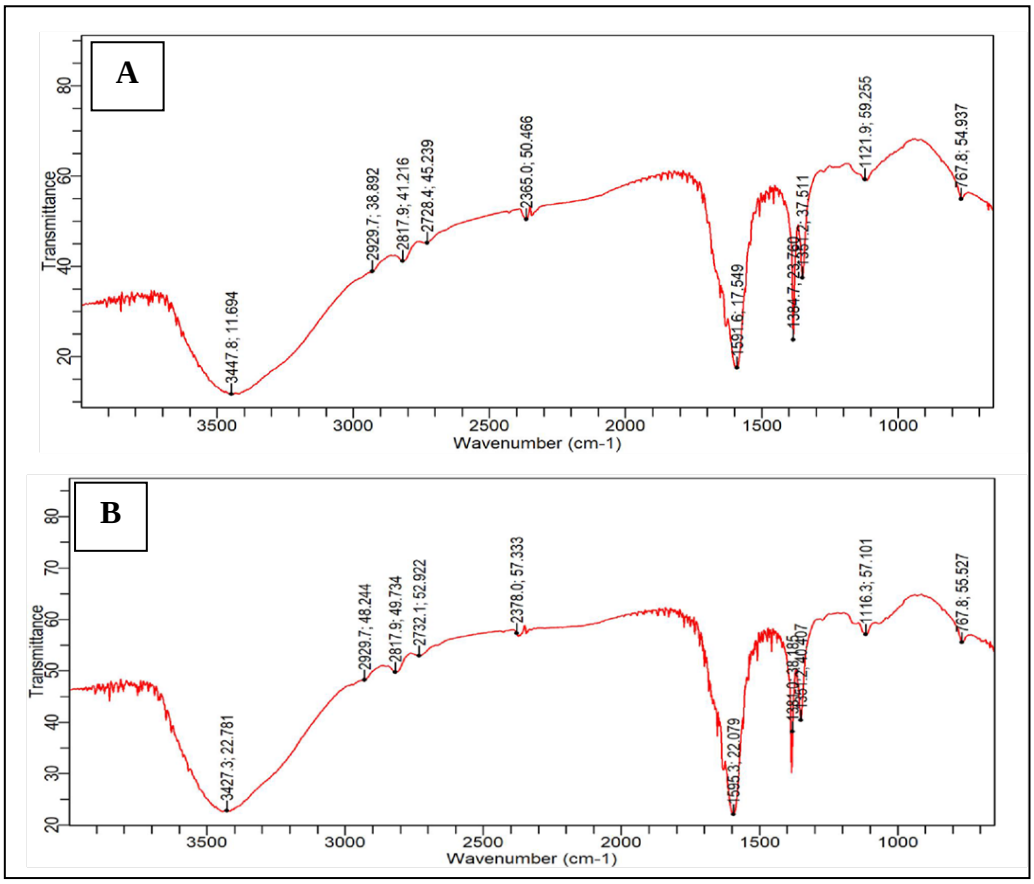


Figure 4.8: (A). FTIR wave numbers of uncoated microparticles, Figure (B). IR wave numbers of coated microparticles.

4.4.7 X-ray diffraction (XRD) analysis

The X-ray diffraction of uncoated and coated microparticles was performed to scrutinize any alters in the drug's physical state during the formulation process. X-ray

diffraction studies of uncoated and coated microparticles showed a peak at 20º, as shown in Figure 4.9 (A). The polymer has amorphous nature. No massive difference was monitored in uncoated and coated microparticles. The uncoated microparticles Figure 4.9 (B) showed diffraction peaks at 2θ; 5.86º,10.56º, 22.26º, 27.38º and 32.89º. However, coated microparticles showed diffraction peaks at 2θ; 5.92º, 10.15º, 22.79º, 27.79º and 32.97º. The XRD peak indicated that the intensity remains almost the same in CAP-coated microparticles, which confirmed no chemical reaction in drug and polymers.

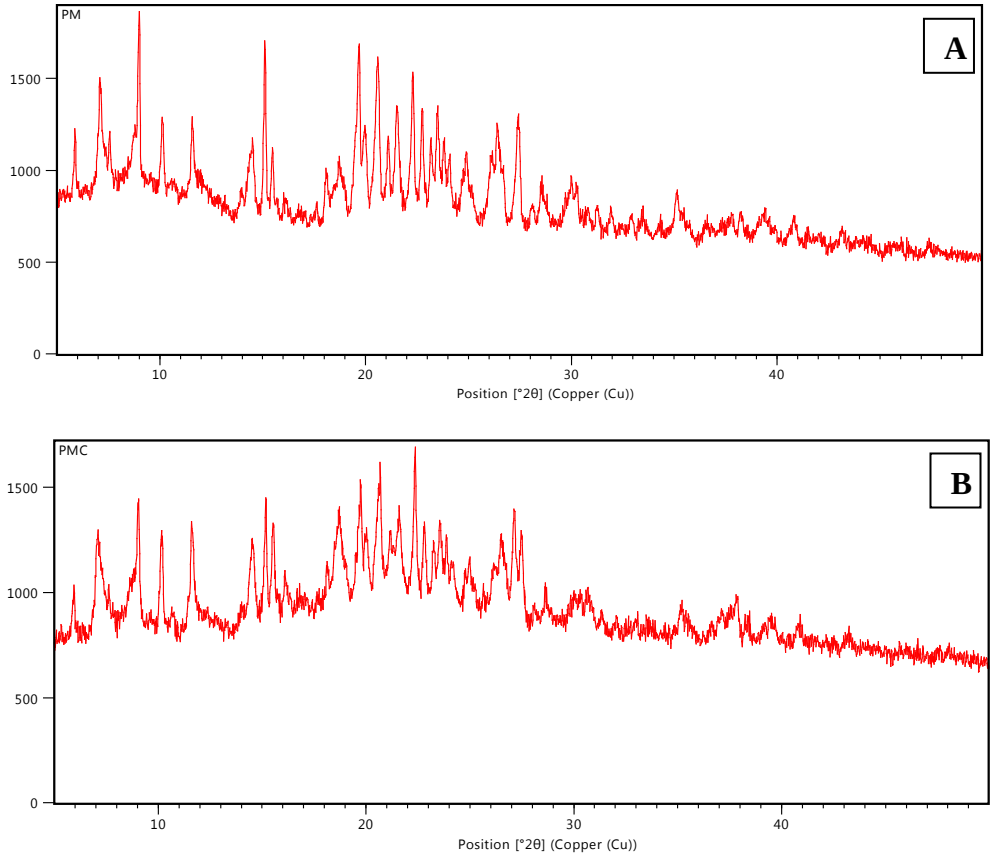


Figure: 4.9 XRD of uncoated (A) and coated microparticles (B).

4.4.8 DSC analysis

DSC is a thermo-analytical method employed to study the phase transition temperature of drug and polymer transitions in the optimized formulation. In the case

of uncoated pellets (Figure: 4.11A). Mesalamine DSC showed a peak at 282 ºC, relatively near its melting point. The MCC peaked at 250 ºC, reasonably close to its melting point. The pectin showed a peak at 182 ºC, near its melting point. From all the peaks, it was confirmed that there was a lack of significant interactions between polymers and drugs. In the case of coated pellets (Figure: 4.12B), DSC of Mesalamine showed a peak at 282 ºC, which is relatively close to its melting point. Similarly, MCC showed a peak at 248 ºC, close to its melting point. The coating material CAP exhibited a peak at 200 ºC and pectin at 166 ºC, coinciding with their melting points. The observed peaks ensured no significant interaction between the drug and polymers.

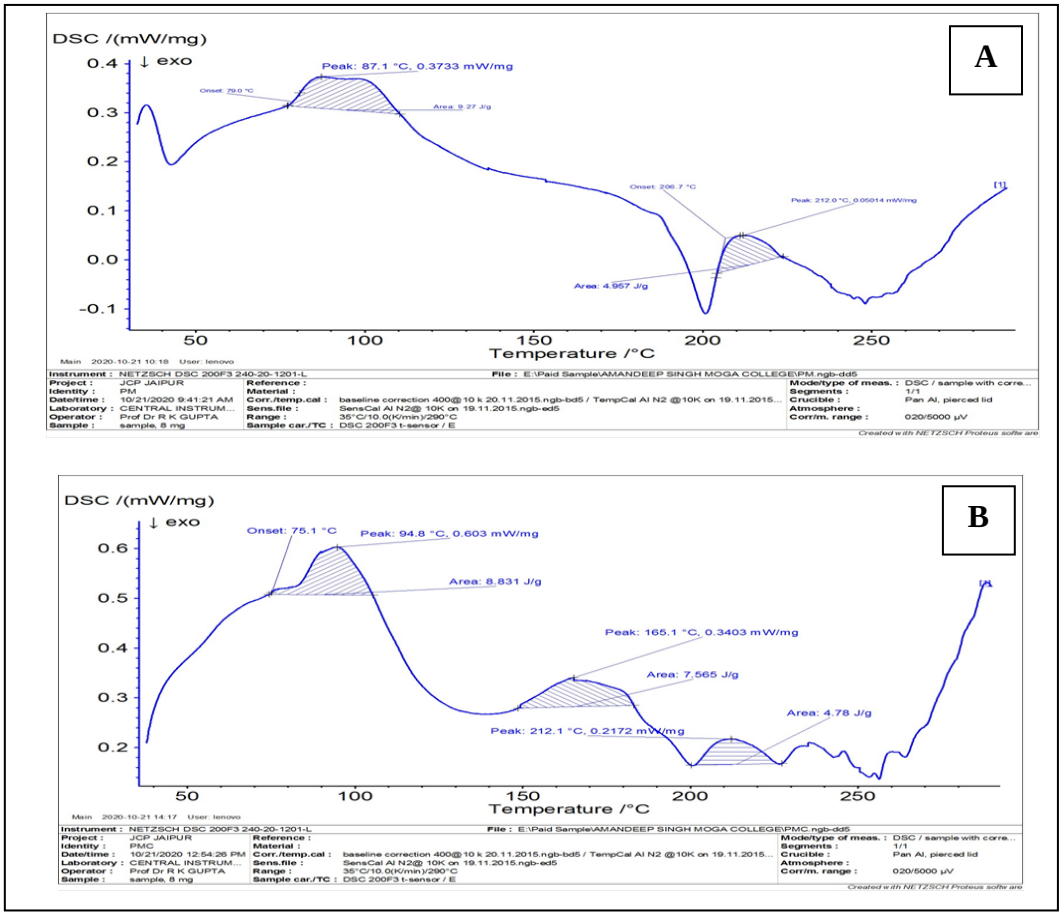


Figure: 4.10 DSC of uncoated (A) and coated microparticles (B)

4.4.9 *In vitro* drug release

This dissolution study was performed to mimic the gastrointestinal tract conditions as our main aim was to prepare colon-targeted drug delivery systems of mesalamine and *S. boulardii*. The prepared uncoated and coated microparticles were placed in different simulated gastrointestinal fluids. In the case of a plain drug, burst drug release was observed in the initial 1 hrs. More than 65% of the drug was released in the first 2 hrs in 0.1 N HCl (SGF) and around 90% drug was released within 6 hrs (SIF). In the case of uncoated microparticles, approximately 40% of the drug release was observed in the first 2 hrs in 0.1 N HCl (SGF). More than 70% of drug release was observed within 6 hrs (SIF). Around 90% drug release was observed within 10 hrs in SCF. In the case of coated microparticles, only 2-3% drug release was observed in the first 2 hrs in 0.1 N HCl (SGF).

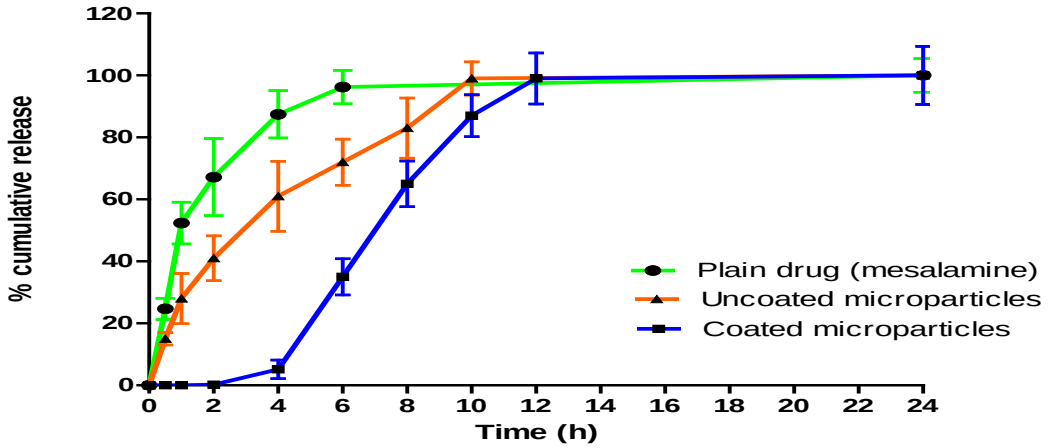


Figure: 4.11 Cumulative % drug release profiles of the plain drug, uncoated and coated microparticles in pH 1.2, phosphate buffer pH 6.8 and 7.4. Data are shown as mean ± SD.

Due to the enteric coating of microparticles, CAP can restrict drug release in the stomach. This small quantity of drug release through this period may be due to the presence of the un-entrapped drug on the surface of the microparticles or diffusion of

the drug from the outer surface of the pectin microparticles. Around 7% drug release was observed within the first 4 hrs. More than 35% of drug release was observed within 6 hrs due to the removal of the coating of CAP in SCF. About 70% of drug releases was observed within 10 hrs. Almost 96% of drug release was accomplished in 24 hrs in SCF at pH 7.4, as shown in Figure 4.11.

4.4.10 *In vitro* probiotic release and viability count

The uncoated microparticles showed that about 50% of probiotics were released in 2 hrs in SGF at pH 1.2. Almost 75% of probiotic was released in SIF (pH 6.8) in 5 hrs of uncoated microparticles. Approximately 90% probiotic release was observed in 10 hrs at pH of 7.4 SCF. Near about 70% of the probiotic release was observed within 8 hrs. In the case of coated microparticle formulation in the first 2 hrs, in SGF at pH 1.2, only 2-4% probiotic release was observed. At the 4th hrs, pH 6.8, about 25% of probiotic releases were observed due to the eruption of coating polymer by SIF. Approximately 96% of probiotic release was observed within 24 hrs, as shown in Figure 4.12.

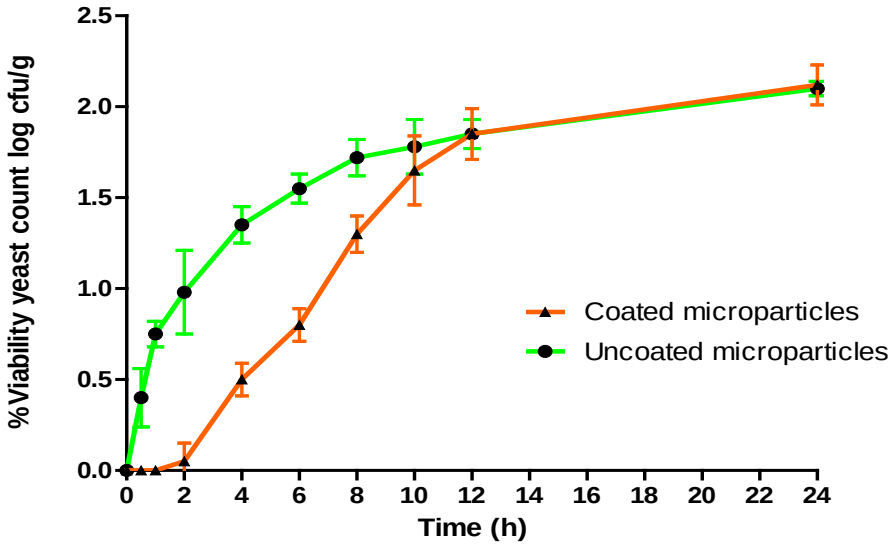


Figure: 4.12 Viability count and release of *S.boulardii* from uncoated and coated microparticles in pH 1.2, pH 6.8, and pH 7.4. Data are shown as mean ± SD.

A slight rise in viable cell count was observed by a combination of drug and probiotic in pectin-based CAP-coated microparticles confirming that this combination can be used further for *in vivo* studies. In SCF presence during the 10^{th} hrs, uncoated and coated microparticles of *S.boulardii* showed $2.03x10^{7}$ and $2.07x10^{7}$ CFU/g viable count, respectively.

4.5 STABILITY STUDY

The stability study of coated microparticles was performed at temperatures (25 ± 2 ºC and 40 ± 2 ºC) and (60 ± 5% as well as 75 ± 5%) respectively, with relative humidity for 6 months. The changes in percentage drug entrapment, moisture content and percentage cumulative drug release of formulations. There was no significant decrease in the percentage of drug entrapment, moisture content and cumulative percentage of drug and probiotic release of formulations indicating high stability of the coated microparticles formulations, as shown in Table 4.12.

Table: 4.12 Stability of coated microparticles loaded with drug-probiotic after 6 months

Coated microparticles	Stability at 25±2 °C				Stability at 40±2 °C			
	0th day	60th day	120th day	180th day	0th day	60th day	120th day	180th day
% Drug entrapment	84.49 ± 3.15	84.21 ± 2.56	83.87 ± 4.51	83.38± 3.67	84.49 ± 3.15	84.31 ± 4.87	84.13 ± 6.23	83.12 ± 5.49
% Moisture content	4.98± 0.36	5.35± 0.56	5.74± 0.18	5.98± 0.36	4.98± 0.36	3.96± 0.48	3.06± 0.31	2.17± 0.43
% Cumulative drug release	75.12± 1.3	74.54± 1.9	74.29± 2.1	73.11± 3.7	75.12 ± 1.3	75.27 ±2.8	74.47± 2.4	74.87± 3.6
% Cumulative probiotic release	95.89± 2.5	95.46± 3.5	95.09± 1.9	94.45± 3.9	95.89 ± 2.5	95.26 ±4.1	94.56± 4.7	94.11± 3.4

4.6 *IN VIVO* STUDIES

4.6.1 Assessment of bodyweight

Compared to the control group, a decrease in Wistar rats' body weight exposed to the treatment schedule for TNBS-induced UC was observed. Each treatment schedule's effects were observed in six animal groups on the 0^{th} day, 7^{th} day and 15^{th} day. Improvement in body weight during the treatment period is considered a sign of the diseased condition's recovery, as shown in Table 4.13. The weight of diseased animals was significantly decreased ($p > 0.001$) compared to the control group. While upon treatment with CAP-coated microparticle formulation, the weight was improved considerably. Bodyweight assessment of different groups. Each data represents mean ±S.D. (n =6). Significance was tested using one-way ANOVA and Tukey–Kramer post-test. ***$p < 0.001$ (Normal control vs. disease control group), $^{ns}p > 0.05$, ##$p < 0.01$ and ###$p < 0.001$ (disease control vs. treatment groups).

Table: 4.13 Bodyweight assessment of different groups

Groups	Treatment given	Body weights (grams)		
		0^{th} Day	**7^{th}Day**	**15^{th}Day**
1	Normal control	243.7±5.31	247.3±6.36	253.5±4.76
2	Disease control	249.6±4.48	239.5±7.21	232.5±5.85***
3	Colitis+ placebo group (Inert material formulation), oral route	248.5±7.58	238.4±4.39	$233.2±4.76^{ns}$
4	Colitis+Mesalamine microparticles (23 mg/kg, oral route)	248.2±3.71	249.4±5.09	$253.1±8.11^{\#\#}$
5	Colitis+probiotic microparticles (10^9 CFU), oral route	248.7±8.38	249.3±6.12	$250.2±7.81^{\#\#}$
6	Colitis+ coated microparticles of Mesalamine (23 mg/kg)+ probiotic (10^9 CFU) for 15 days, by oral route	245.3±5.27	253.3±8.21	$257.2±6.33^{\#\#\#}$

4.6.2 Macroscopic activity score

The TNBS model of colitis has been used to induce colon inflammation. The percentage change in weight, stool consistency, lesion score, and macroscopic scores for the colitis group was observed to be 4 ± 0.07, 4± 0.04, 4± 0.07 and 4± 0.09 correspondingly. Whereas for CAP-coated microparticles, the scores for the percentage change in weight, stool consistency, score of the lesion and macroscopic scores for the colitis group were found to be 1± 0.3, 1± 0.08, 1± 0.13, 1± 0.21 correspondingly. The Wistar rats' weight was measured at the beginning and end of the treatment schedule (15^{th} day), as shown in Table 4.14.

Table: 4.14 Macroscopic parameters assessment to evaluate disease; values are statistically significant at p<0.05 compared to normal and p<0.05 compared to disease control animals (n=3).

Treatment	Scores (% weight loss)	Stool Consistency	Lesion Scores	Macroscopic Scores
Normal control	0.0 ± 0	0.0 ± 0	0.0 ± 0	0.0 ± 0
Disease control	4 ± 0.07	4± 0.04	4± 0.07	4± 0.09
Colitis+ placebo group (Inert material formulation), oral route	0.0 ± 0	0.0 ± 0	0.0 ± 0	0.0 ± 0
Colitis+Mesalamine microparticles (23 mg/kg), oral route	2 ± 0.51	2 ± 0.31	3 ± 0.24	3 ± 0.16
Colitis+probiotic microparticles (10^9 CFU), oral route	3 ± 0.25	3 ± 0.48	3 ± 0.76	3 ± 0.59
Colitis+ coated microparticles of Mesalamine (23 mg/kg)+ probiotic (10^9 CFU) for 15 days, by oral route	1± 0.3	1± 0.08	1± 0.13	1± 0.21

4.6.3 Diarrhoea assessment during the treatment period

Rank 1 for moderate to mild diarrhea was observed in group I rats for the initial 1-2 days after drug delivery of TNBS. There was a significant change in the stool consistency during the treatment period in rats group II, IV, V and VI. After administration of coated Mesalamine and probiotic pellets to the rats, huge improvement towards normal consistency of fecal and reduced bleeding was observed from the 4th day onwards, as depicted in Figure 4.13. Normal stool consistency (Rank 0) lacking any traces of blood was attained in all the groups at the end of the treatment period.

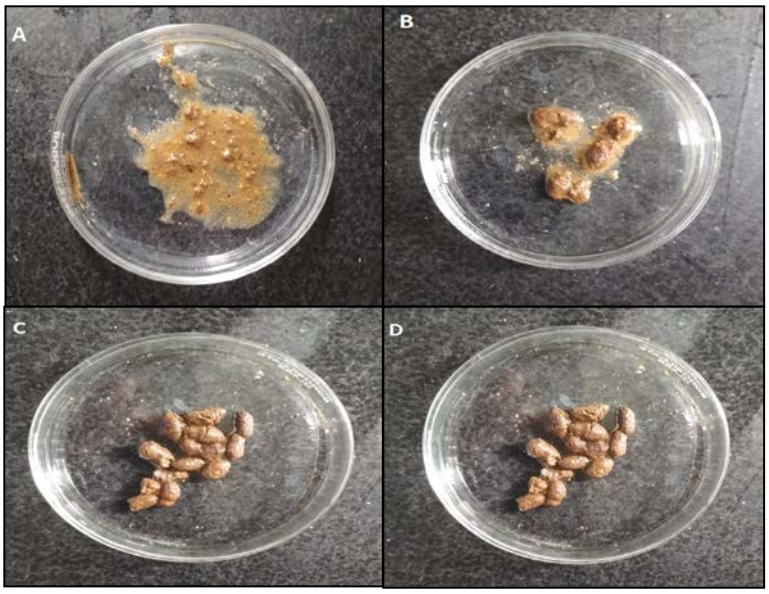


Figure: 4.13 Stool consistencies on different days of treatment A) during 2 days UC induction period, B) after 5 days of the treatment period, C) & D) at the end of the treatment period.

4.6.4 Effect of microparticles formulation on MPO in TNBS-induced colitis in Wistar rats

Due to the colitis induction, the colonic enzyme concentration becomes changed. MPO is a colonic enzyme. This enzyme's activity concerns the concentration of neutrophils in the inflamed tissue. Therefore, colonic enzyme determination can be a

parameter for assessing acute intestinal inflammation. TNBS via intrarectal administration showed a significant MPO concentration increase, i.e., 19.06 µ mol/min/mg tissues, while the MPO concentration was almost the same in the normal and placebo groups. Plain Mesalamine microparticles reduce MPO concentration, i.e., 11.6 µmol/min/mg, which is near the value obtained after giving plain probiotic microparticles, i.e., 13.2 µmol/min/mg. However, coated microparticles provide a remarkable reduction in MPO concentration, i.e., 7.9 µmol/min/mg, compared to disease control, as shown in Figure 4.14.

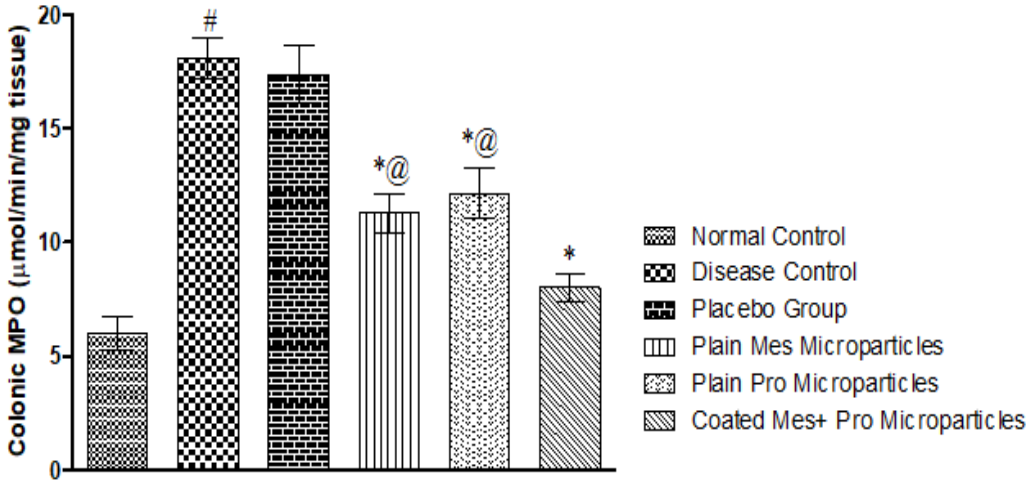


Figure: 4.14. Effect of microparticle formulation on MPO) in TNBS induced colitis in Wistar rats. Values are given as mean± SD; values are statistically significant at $p<0.005$.$^{\#}p<0.001$ vs normal, $^{*}p<0.01$ vs disease control,and $^{@}p<0.05$ vs coated mes+ pro microparticles.

4.6.5 Effect of microparticles formulation on LPO in TNBS-induced colitis in Wistar rats

LPO is a colonic enzyme. An increase in LPO concentration can lead to reactive metabolites, which can further cause inflammation. TNBS via intrarectal administration demonstrated a considerable rise in LPO concentration in the disease control group, i.e., 153.11 µ mol of MDA/mgpr, while the placebo group had almost

the same LPO concentration in comparison with normal control. Plain Mesalamine microparticles reduce LPO concentration, i.e., 125.16 µ mol of MDA/mgpr. The effect of plain probiotic microparticles 129.7 µ mol of MDA/mgpr. However, CAP-coated microparticles of Mesalamine with probiotics give a remarkable decrease in the LPO concentration up to 74.37 µ mol of MDA/mgpr compared to disease control, as shown in Figure 4.15.

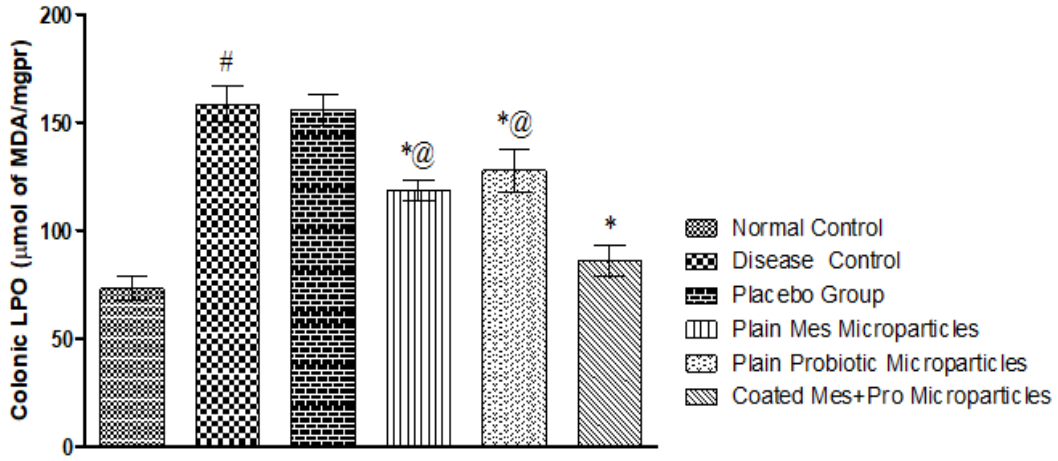


Figure 4.15 Effect of microparticle formulation on LPO in TNBS-induced colitis in Wistar rats. Values are given as mean± SD.; values are statistically significant at $p<0.05$. $^{\#}p<0.001$ vs normal, $^{*}p<0.01$ vs disease control, and $^{@}p<0.05$ vs coated mes+ pro microparticles.

4.6.6 Effect of microparticles formulation on GSH in TNBS-induced colitis in Wistar rats

GSH is a colonic enzyme involved in DNA repair and is also responsible for antioxidant activity. Intrarectal administration of TNBS has shown an extensive decrease in GSH concentration in the disease control group, i.e., 4.1 µ mol of GSH/mgpr, nearly the same as in the placebo group. Plain Mesalamine microparticles increase the GSH concentration, i.e., 5.4 µ mol of GSH/mgpr, but probiotics microparticles raise the GSH concentration, i.e., 4.7 µ mol GSH/mgpr, which was lesser than the Mesalamine effect. CAP-coated mesalamine microparticles loaded

with probiotics showed an impactful increase in GSH concentration, i.e., 6.2 µ mol of GSH/mgpr compared to disease control, as shown in Figure 4.16.

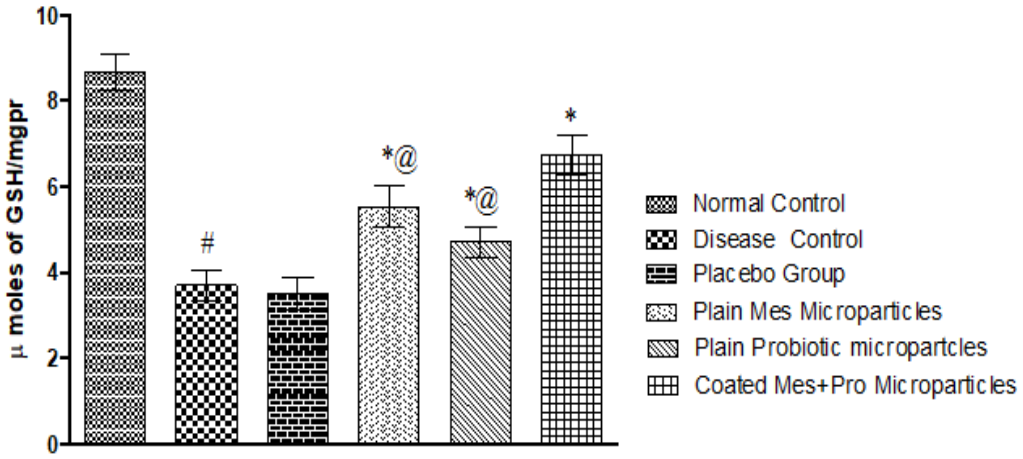


Figure: 4.16 Effect of microparticle formulation on GSH in TNBS-induced colitis in Wistar rats. Values are given as mean± SD.; values are statistically significant at p<0.05as compared to normal and p<0.05 as compared to disease control rats.

4.6.7 Determination of C-reactive protein

CRP is a sensitive marker of inflammation; Table 4.15 showed that CRP levels are increased in UC, but treatment with CAP-coated mesalamine and probiotic microparticles showed a reduction in CRP levels very effectively. Mesalamine microparticles as well as plain probiotic microparticles. However, the level of CRP was almost similar for disease control and placebo.

4.6.8 Determination of ESR

Erythrocyte sedimentation rate (ESR) is an easy test for assessing inflammatory response. The observed results have shown that CAP-coated microparticles of Mesalamine and probiotics have the property to considerably reduce the ESR compared with plain Mesalamine microparticles and plain probiotic microparticles.

However, the ESR level was similar to disease control and the placebo, as shown in Table 4.15.

4.6.9 Determination of WBC

The body releases white blood cells when an infection or inflammatory disease arises to help fight the infection. As shown in Table 4.15, WBC levels are increased after UC due to the cells' proliferation. However, coated microparticles of Mesalamine and probiotics have shown a marked reduction in the level of WBCs in comparison with plain microparticles of mesalamine and probiotics, respectively. However, the results are almost identical for the placebo and disease control groups.

Table: 4.15 Determination of WBC, CRP and ESR levels after treatment schedule

Group	WBC (µl)	CRP(mg/dl)	ESR(mm/hr)
Normal control	$9.9 \pm 0.18 \times 10^3$	2.7 ±0.12	3.2 ±0.32
Disease control	$14.9 \pm 0.26 \times 10^3$	10.1 ±0.68	23.2 ±1.18
Colitis+ placebo group (Inert material formulation), oral route	$14.8 \pm 0.46 \times 10^3$	9.7 ±0.42	21.9 ±1.06
Colitis+Mesalamine microparticle (23 mg/kg, oral route)	$12.4 \pm 0.38 \times 10^3$	8.6 ±0.38	11.9 ±0.76
Colitis+probiotic microparticles (10^9 CFU), oral route	$12.6 \pm 0.76 \times 10^3$	8.8 ±0.62	14.8 ±0.92
Colitis+ coated microparticles of Mesalamine (23 mg/kg)+ probiotic (10^9 CFU) for 15 days, by oral route	$10.1 \pm 0.92 \times 10^3$	5.7 ±0.24	8.8 ±0.54

4.7 PHARMACOKINETICS ESTIMATION

The concentration against time profiles after administration via oral the uncoated and coated microparticles has been presented in Figure 4.17. After oral administration of uncoated microparticles, T_{max} was found to be 3.1±0.17, which was significantly different ($p<0.05$) from the 8.11±0.55 T_{max} obtained from the coated microparticles. The observed mean C_{max} for the uncoated microparticles (12.13±0.52 mg/ml) was much higher than that obtained from the coated microparticles (5.91±0.69 mg/ml). The determined pharmacokinetics parameters have been given in Table 4.16. The MRT value of the drug obtained from the uncoated microparticles was about 4.3 hrs, much lower than that of the coated microparticles (12.34 hrs). The AUC from the uncoated microparticles (34.45±1.38 µg/ml/h) was lower than that obtained from the coated microparticles (63.32±1.62µg/ml/h). Pharmacokinetic outcome found the controlled drug release manners of the optimized microparticles formulation, which recommends more capable management of colitis by supplying more drug concentration at the colonic site and decreasing systemic drug absorption.

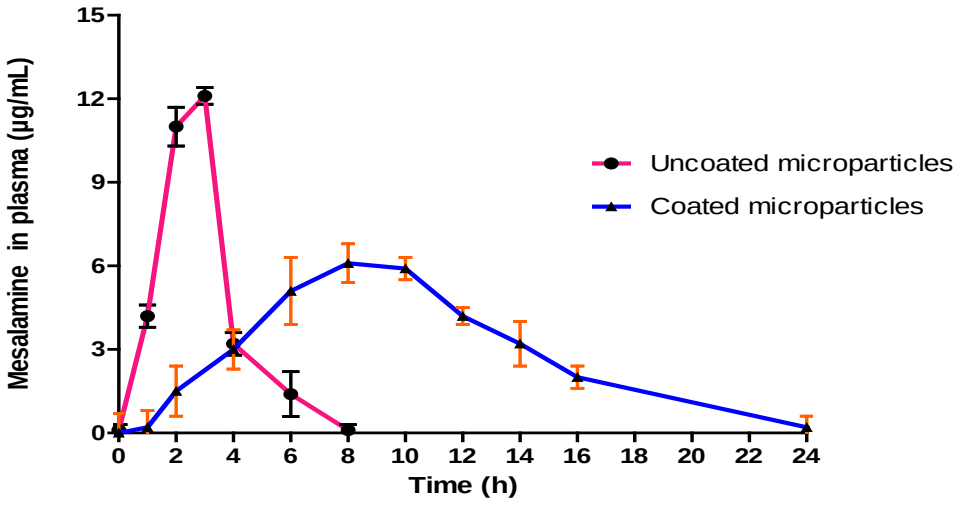


Figure: 4.17 Plasma concentration profiles of Mesalamine after oral administration of uncoated and coated microparticles in Wistar rats. Data are shown as mean±SD (n=3).

Table: 4.16 Pharmacokinetic parameters of Mesalamine after oral administration of uncoated and coated microparticles

Parameters	Uncoated microparticles	Coated microparticles
C_{max} (mg/ml)	12.1	5.9
T_{max} (h)	3.1	8.1
AUC_{total} (µg/ml/h)	34.45	63.32
$T_{1/2}$	3.1	8.5
MRT (h)	4.3	12.34

4.8 HISTOPATHOLOGY EXAMINATION

Histological examination of tissues can help diagnose disease conditions because each condition causes characteristic changes in the tissue structure. Histopathology studies (Figure 4.18) have shown that in the colon tissue of Group I (A), the mucosal lining appears normal, showing a normal ionic gland with minimal stroma with goblet cells and no infiltration, muscularis mucosae and normal submucosa. Group II (B)'s colon tissue revealed histopathological changes, including severe mucosal ulceration replacement by inflammatory cellular infiltrate and fibrinoid-like necrosis in all colonic wall layers. There was a marked rise in the number of inflammatory cells, mainly in lamina propria, consisting of lymphocytes with lymphoid follicle formation- neutrophils, eosinophils–goblet cell, depletion distribution mucosal gland featuring. Group III (C)'s colon tissue showed an increase in inflammatory cell number in lamina propria, with the invasion of the base of crypts and progress in the direction of crypt lumina to form crypt abscess, eosinophils –goblet cell, depletion and distribution mucosal gland featuring. Group IV (D) expressed better reemission and a slight improvement in the infiltration of cells has been observed. In Group V (E), orally administered plain probiotic microparticles showed a small change in histopathological conditions. In Group VI (F), orally administered coated microparticles of Mesalamine and probiotic histopathological examination revealed

intact mucosal lining with mucosal line gland and showed almost 100% recovery of colonic mucosa from TNBS-induced colitis damage in comparison with other groups. Especially coated microparticles of mesalamine and probiotics showed the advantages of synergistic effects for colitis rats.

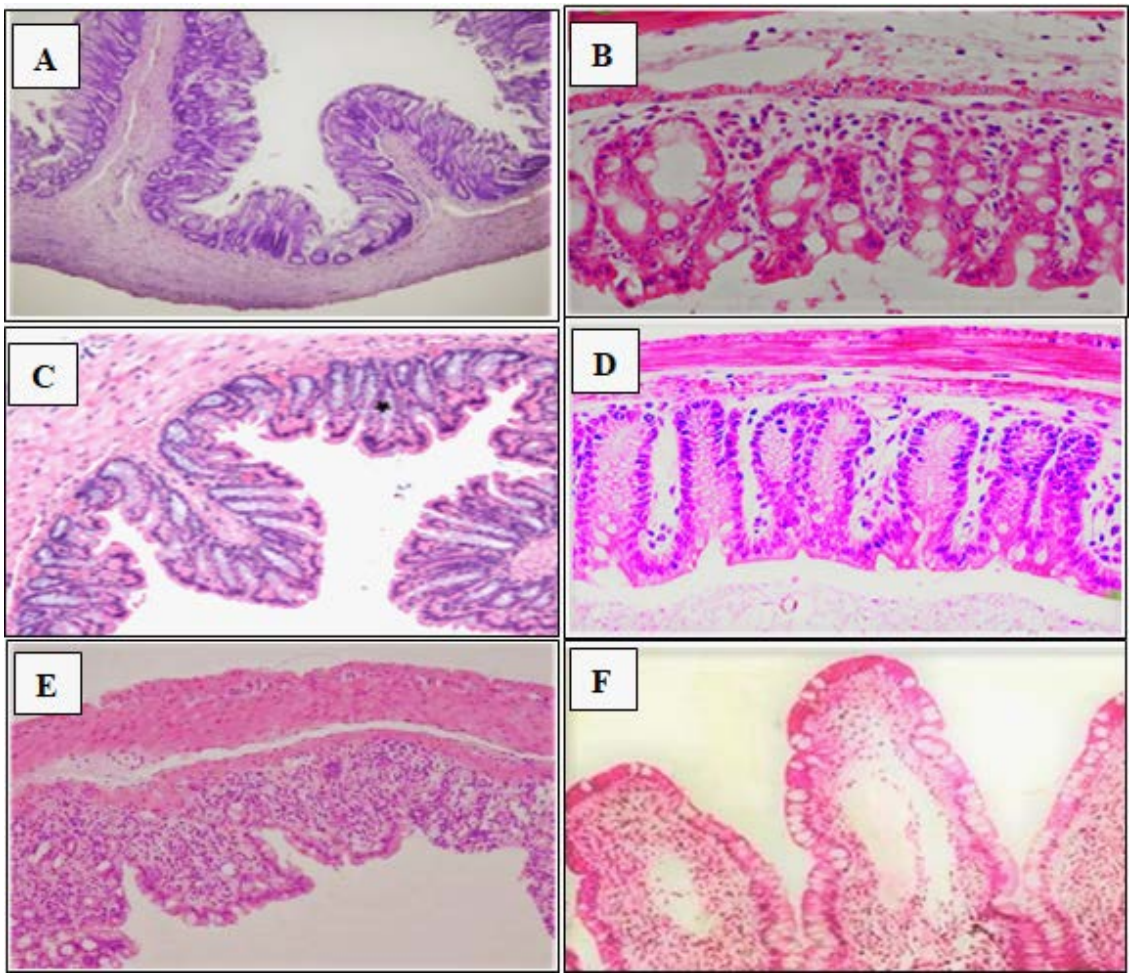


Figure 4.18: Histopathological examination of colonic sections (x200); A- Normal control, B- Disease control, C- Placebo group, D- Plain Mesalamine microparticles (23 mg/kg), E-Plain probiotic microparticles (10^9 CFU), F-Coated microparticles of Mesalamine +probiotic (10^9 CFU), through oral administration for 15 days.

4.9 PELLETS FORMULATION RESULTS

4.9.1 Preparation and optimization of uncoated drug and probiotic-loaded pellets

Process variables, i.e., pectin and MCC weight ratios, were finalized according to their effect on percentage yield, moisture content and the shapes of pellets. The extrusion-spheronization method was used to formulate using water as a wetting agent. For the first batch (MP1), the pectin concentration (16%) and MCC concentration (40%) were used (Table 4.17). The results showed that as the pectin concentration increased from 16% to 26%, where the concentration of MCC was constant (40%) (MP5), a decrease in pellets size and an increase in percentage yield were observed.

Table: 4.17 Composition of pellet formulations

Formulation coding	Probiotic (CFU/ml)	Pectin (%)	MCC (%)	Drug (%)
MP1	10^9	16	40	1
MP2	10^9	18	40	1
MP2	10^9	20	40	1
MP3	10^9	22	40	1
MP4	10^9	24	40	1
MP5	10^9	26	40	1
MP6	10^9	28	50	1
MP7	10^9	28	60	1
MP8	10^9	28	70	1
MP9	10^9	30	70	1
MP10 (uncoated)	10^9	29	70	1
MP11(coated)	10^9	29	70	1

However, the percent yield increased when the pectin and MCC concentration was 29% and 70%, respectively. In these concentrations, the pellets showed maximum roundness and sphericity factor. A decrease in entrapment efficiency was observed when more than 70% MCC was used and the pectin concentration was constant (29%). After obtaining the desired results (Moisture content: 4.33±0.04, Percentage yield: 86.33±0.19, Average particle size: 518±20.2 µm), the bath MP10 was selected for the further coating process and tagged as MP11 (Table 4.18).

Table: 4.18 Optimization of rotation speed and time of spheronization process

MP1, MP2, MP3, MP4, MP5, MP6, MP9 & MP10		**MP7 & MP8**	**MP7**	**MP8**
Rotation speed (rpm)	**Rotation time (min.)**	**Rotation speed (rpm)**	**Rotation time (min.)**	**Rotation time (min.)**
400	2	500	5	10
700	4	200	10	10
700	4	300	10	15
700	4	700	10	15
700	4	700	10	15

After optimization of micrometric properties, MP11 has been coated with CAP. Four different concentrations, i.e., 2.5%, 5%, 7.5% and 10% of CAP, were used, as shown in Table 4.19. The 2.5% concentration of CAP fluctuated drug release has been observed. The best coating targeted and controlled drug and probiotic release were at 5% CAP w/v. At 7.5% concentration of CAP the solution became more viscous and droplets were formed on the tip of the atomizer.

The concentration of CAP of more than 10% was challenging to pass through the atomizer due to the formation of aggregates 5% CAP concentration of coating material provides a smooth surface with desired *in vitro* drug and probiotic release (Figure 4.19).

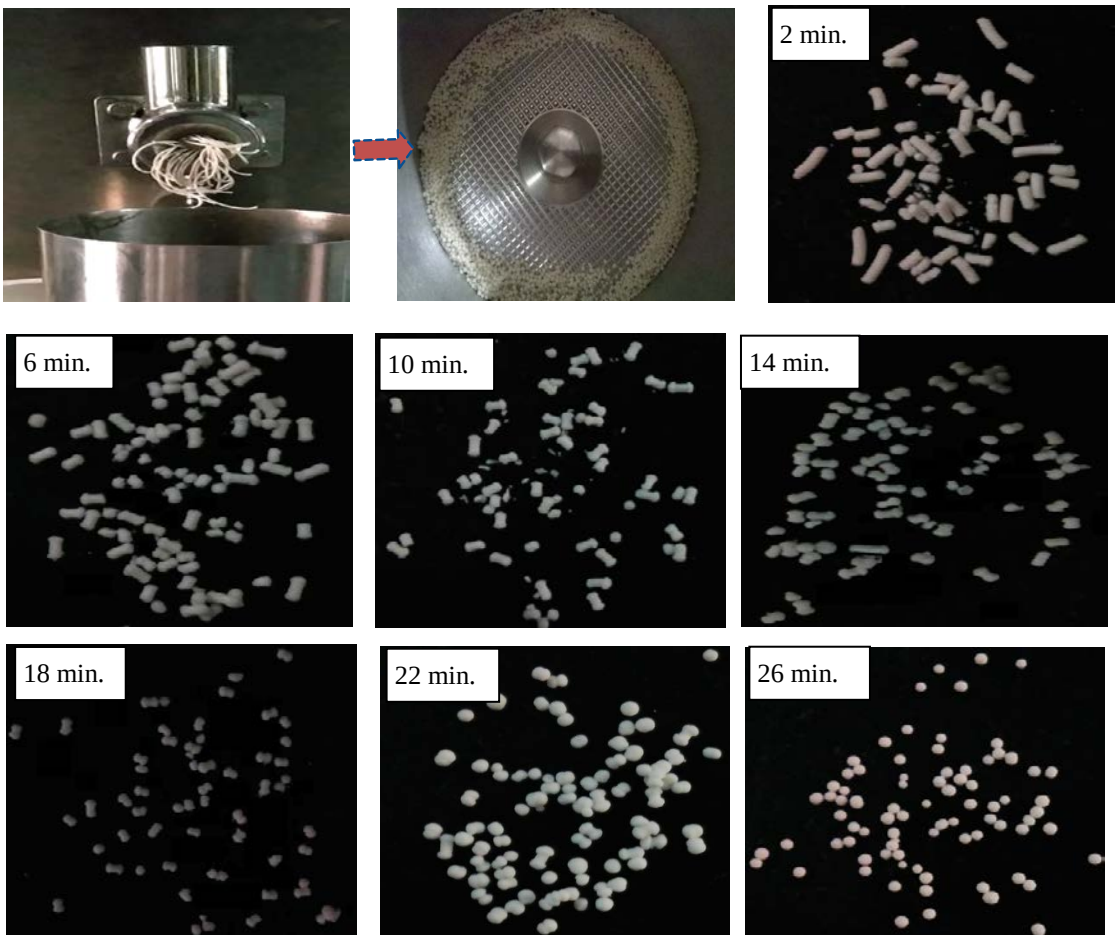


Figure: 4.19 In-process images of pellets at different time intervals.

Table: 4.19 Optimization of variables for coated pellets

CAP Concentration (%)	Flow rate (ml/min)	Drum speed (rpm)	Temperature (^oC)	Air pressure (kg/cm^2)	Conclusion
2.5	1.5	20	55	5	Slight variation *in vitro* drug release profile
5	1.5	20	55	10	Desired *in vitro* drug release
7.5	1.5	20	55	10	Bead formation at the tip of the atomizer
10	1.5	20	55	10	Difficulty in passing from the atomizer

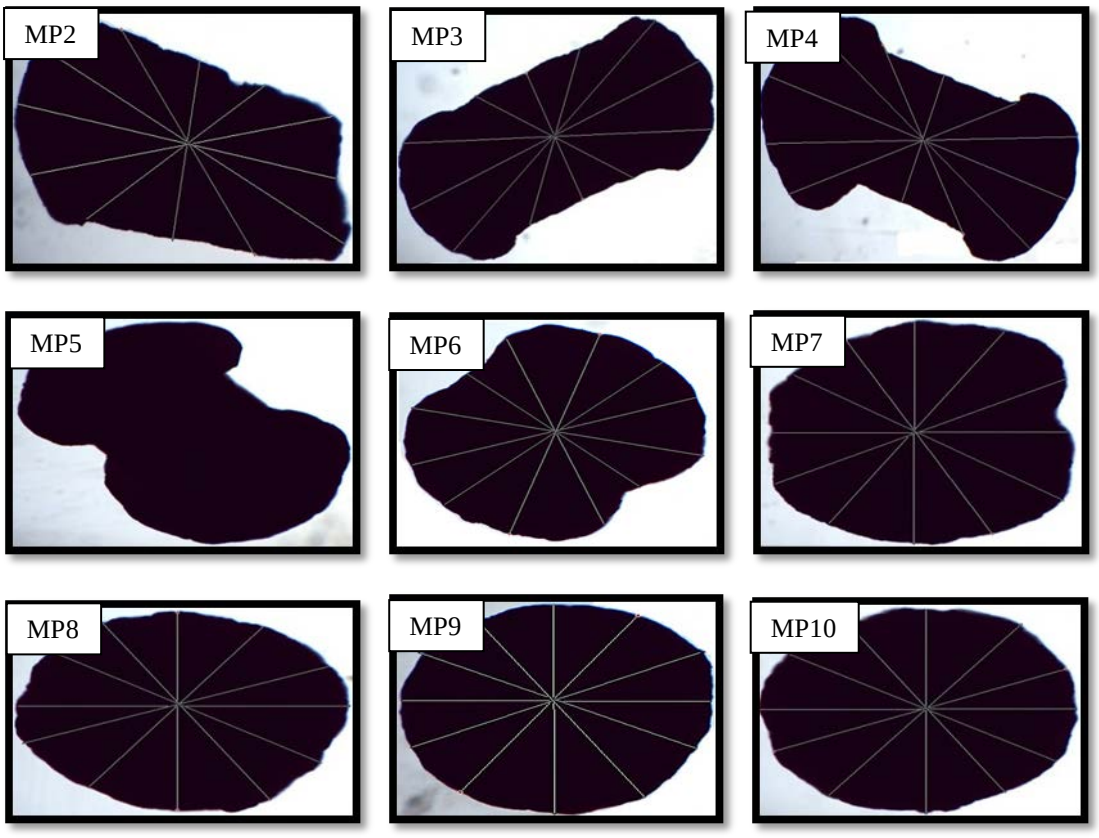
4.10 *IN VITRO* CHARACTERIZATION OF DRUG-PROBIOTIC-LOADED PELLETS

4.10.1 Drug loading and entrapment efficiency

Drug-loaded pellets entrapment efficiency was determined. The percentage drug loading and entrapment efficiency was 1% and 82.67%±0.05 for coated pellets. A slight loss in entrapment efficiency in coated bullets ascribed to the loss of drug-related to coating polymer would stick on the coating surface throughout the coating process.

4.10.2 Pellets morphology

The mean particle size of pellets, as shown below in Figure 4.20 of optimized pellets of MP9, MP10 and MP11, was found to be 500 µm, 480 µm and 530 µm, respectively.



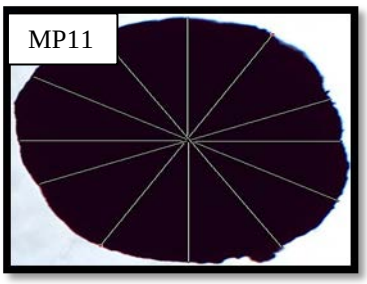


Figure: 4.20 Mean particle size of optimized pellets: A) MP2, B) MP3, C) MP4, D) MP5, E) MP6, F) MP7, G) MP8, H) MP9, I) MP10 Drug loaded uncoated pellets and MP11, J) drug loaded coated pellets.

4.11 SPHERICITY STUDIES

Pellets with a sphericity value of 1 are considered to be precisely spherical and pellets with a sphericity value near one are considered nearly spherical. The aspect ratio, roundness, and circularity factor for the optimized formulation MP 9 were found to be 1.120±0.19, 1.0015±0.13 and 0.988±0.27, respectively (Table 4.20).

Table: 4.20 Particle shape analysis for pellets formulation

Formulation Code	Shape	Aspect ratio	Roundness Factor	Circularity factor
MP1	Rod-shaped	1.359±0.15	1.059±0.11	0.964±0.39
MP2	Rod-shaped	1.325±0.21	1.050±0.25	0.969±0.22
MP3	Rod-shaped	1.305±0.11	1.047±0.29	0.971±0.27
MP4	Dumbbell	1.286±0.23	1.045±0.06	0.975±0.18
MP5	Dumbbell	1.272±0.21	1.039±0.17	0.976±0.21
MP6	Elongated spheroids	1.245±0.16	1.024±0.10	0.977±0.15
MP7	Elongated spheroids	1.230± 0.24	1.021±0.23	0.979±0.26
MP8	Elongated spheroids	1.225± 0.21	1.019±0.19	0.981±0.10
MP9	Spheroids	1.120± 0.19	1.0015±0.13	0.988±0.27
MP10	Spheroids	1.109± 0.24	1.0009±0.11	0.988±0.16
MP11	Spheroids	1.102± 0.09	1.0005±0.09	0.990±0.09

4.12 MICROMETRIC AND OTHER PROPERTIES

Flow properties results, including angle of repose, Bulk density, Tapped density, Hausner's ratio and Carr's index of different formulations of pellets, are given in Table 4.21. The angle of repose (θ^o) for the uncoated and coated pellets were found to be 25.116±0.341 and 24.213±0.361 respectively, which has shown excellent flow properties of mesalamine and probiotic-loaded pellets formulations. The friability test was conceded for optimized formulations to ensure their mechanical strength. The MP10 and MP11 formulations showed admirable mechanical strength (Table 4.21).

Karl Fischer's (KF) Coulometric titration method was used to assess the percentage of moisture contents of optimized pellets. The percentage of moisture and percentage yield results are shown in Table 4.21.

4.13 MUCOADHESIVE EVALUATION

The quantitative attachment of pellets on excised intestinal tissue has been presented in Figure 4.21. A huge pellets number on the mucosa specified an enhanced adhesion of the pectin-based pellets. Approximately 100% of pellets got adhered to the mucosa. The maximum adhesion ability of uncoated pellets on rat mucosa showed that the pectin pellets formulation has good mucoadhesion properties.

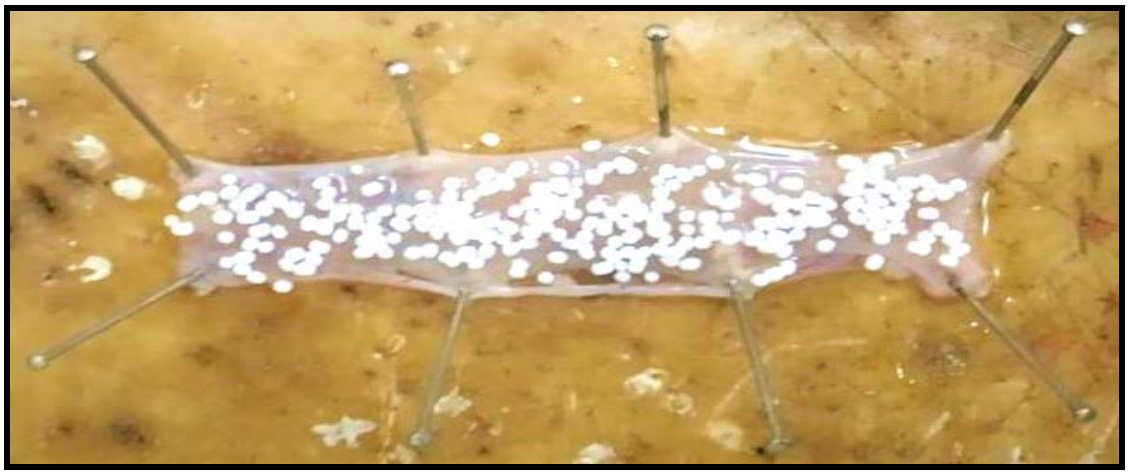


Figure: 4.21 Mucoadhesive properties estimation of uncoated pectin pellets on the mucosa of the rat colon.

Table: 4.21 Micromeritic and other properties of pellets formulation

Batch code	Angle of repose	Bulk density (g/cm^3)	Tapped density (g/cm^3)	Hausner's ratio	Carr's index (%)	Friability (%)	Yield (%)	Moisture content (%)	Average particle size(µm)
MP1	36.250±0.411	0.714±0.004	0.816±0.005	1.134±0.03	20.713±0.207	0.58±0.321	82.17±9.13	3.21±0.01	725± 24.2
MP2	34.425±0.408	0.708±0.003	0.833±0.020	1.203±0.03	20.042±0.325	0.56±0.356	82.45±0.17	3.23±0.01	642±19.1
MP3	32.221±0.292	0.723±0.040	0.839±0.030	1.077±0.01	17.195±0.348	0.69±0.219	82.26±0.14	3.41±0.03	627±22.7
MP4	31.841±0.360	0.741±0.007	0.839±0.005	1.022±0.04	14.137±0.282	0.89±0.236	82.12±0.19	3.43±0.02	719 ±21.6
MP5	31.920±0.469	0.740±0.060	0.859±0.092	1.198±0.05	13.753±0.156	0.93±0.471	83.19±0.21	4.11±0.03	660± 20.6
MP6	30.842±0.491	0.749±0.003	0.873±0.040	1.155±0.07	13.268±0.441	0.71±0.510	84.12±0.03	4.15±0.04	710± 22.3
MP7	27.219±0.415	0.769±0.002	0.879±0.020	1.206±0.0.6	12.421±0.379	0.68±0.468	84.22±0.08	4.20±0.02	653±17.2
MP8	26.941±0.423	0.786±0.004	0.830±0.030	1.197±0.06	11.415±0.310	0.55±0.402	84.40±0.21	4.21±0.03	541± 18.3
MP9	25.151±0.475	0.773±0.003	0.843±0.020	1.103± 0.03	9.216±0.541	0.46±0.458	86.15±0.13	4.26±0.04	463± 16.3
MP10	25.116±0.341	0.786±0.004	0.846±0.030	1.095±0.04	9.181±0.391	0.55±0.343	86.33±0.19	4.31±0.03	518 ±20.2
MP11	24.213±0.361	0.776±0.005	0.840±0.020	1.084±0.03	8.516±0.562	0.49±0.381	86.21±0.12	4.33±0.04	513 ±19.1

4.14 COMPATIBILITY STUDY THROUGH IR SPECTROSCOPY

The compatibility of the drug and excipients was assessed through IR spectroscopy. The principle IR peaks for mesalamine were found at wavenumbers (cm^{-1}) 3447.8 (O-H stretch), 1591.6 (N-H stretch), 2929.7 (C-H stretch), 1121.9 (C-O stretch), 1384.7 (O-H bend), 1384.7 (C-C stretch), 1121.9 (in-plane bending) and 767.8 (C-H out of plane bending) as depicted in (Figure 4.22: A).

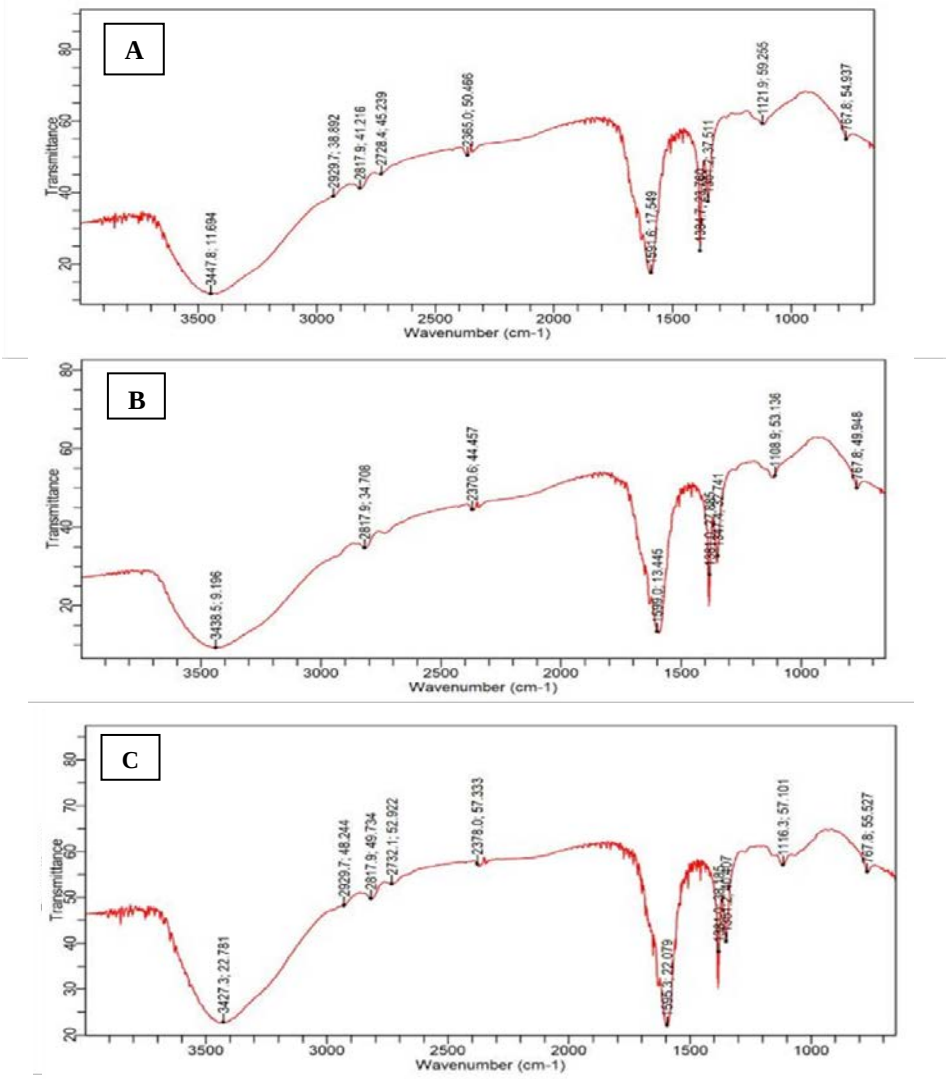


Figure 4.22: Figure (A). FTIR wave numbers of Mesalamine, Figure (B) FTIR wave numbers of uncoated pellets, Figure (C). FTIR wave numbers of coated pellets.

Similarly, the vibrational frequency of uncoated pellets containing Mesalamine, pectin, MCC (Figure 4.22 B) and coated pellets containing mesalamine, pectin, MCC, CAP (Figure 4.22 C) was also analyzed through FTIR spectroscopy. It was evident from the results that all the major peaks were present in FTIR spectra of uncoated as well as coated pectin-based mesalamine and probiotic-loaded pellets. These findings indicate no primary interface between the drug and used excipients. There was no sign of any degradation.

4.15 XRD ASSESSMENT

X-ray diffraction studies of uncoated and coated drug and probiotic-loaded pellets showed peaks at 20ºθ, as shown in Figure 4.23(A). The polymer has amorphous nature.

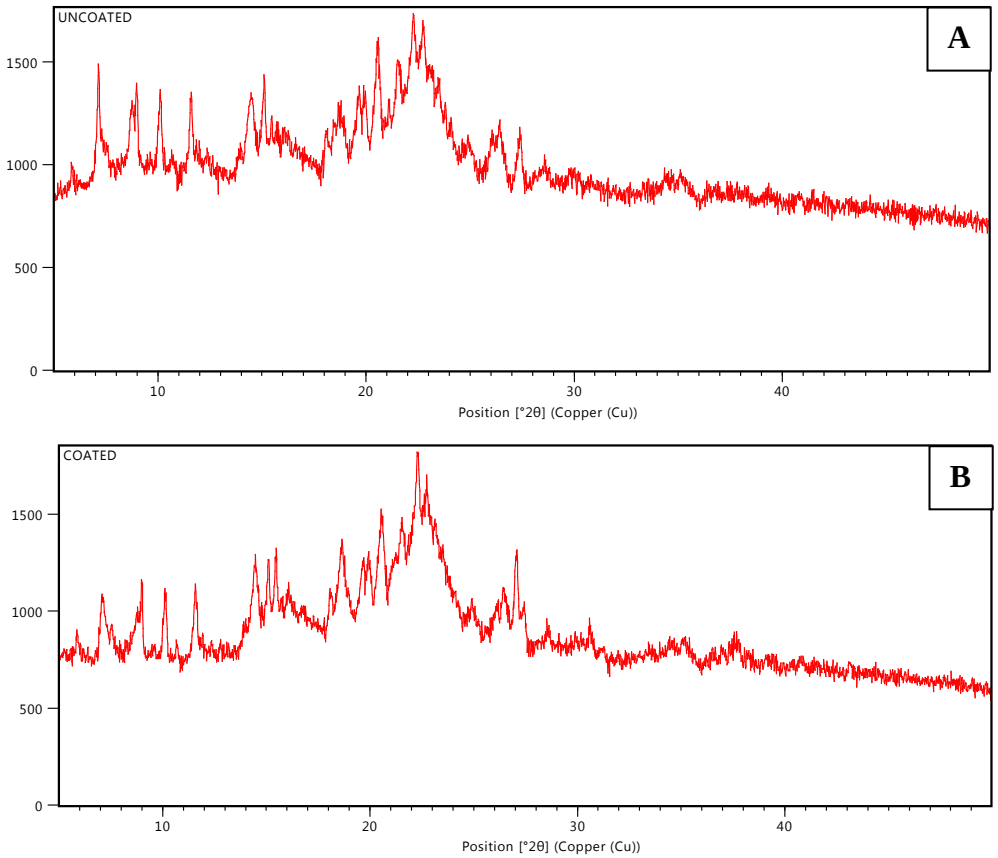


Figure 4.23: XRD of uncoated (A) and coated (B) pellets.

The results showed that the intensity remains almost the same in CAP-coated microparticles, which confirmed no chemical reaction in drug and polymers and no huge difference was observed in uncoated and coated pellets. Uncoated pellets showed diffraction peaks at 2θ; 5.85º, 10.09º, 22.24º, and 26.27º. However, coated pellets Figure 4.23 (B) showed diffraction peaks at 2θ; 5.86º, 10.67º, 22.26º, 26.42º. The peaks in XRD showed that uncoated Mesalamine pellets appear amorphous after coating with CAP. The peaks' intensity remains almost the same, which evidences that there is no chemical interaction between the drug and polymers

4.16 DSC ANALYSIS

DSC is a thermo-analytical method, employed to study the phase transition temperature of drug and polymer transitions in the optimized formulation. In the case of uncoated pellets (Figure 4.24 A). Mesalamine DSC showed a peak at 282 ºC, relatively near its melting point. The MCC peaked at 250 ºC, close to its melting

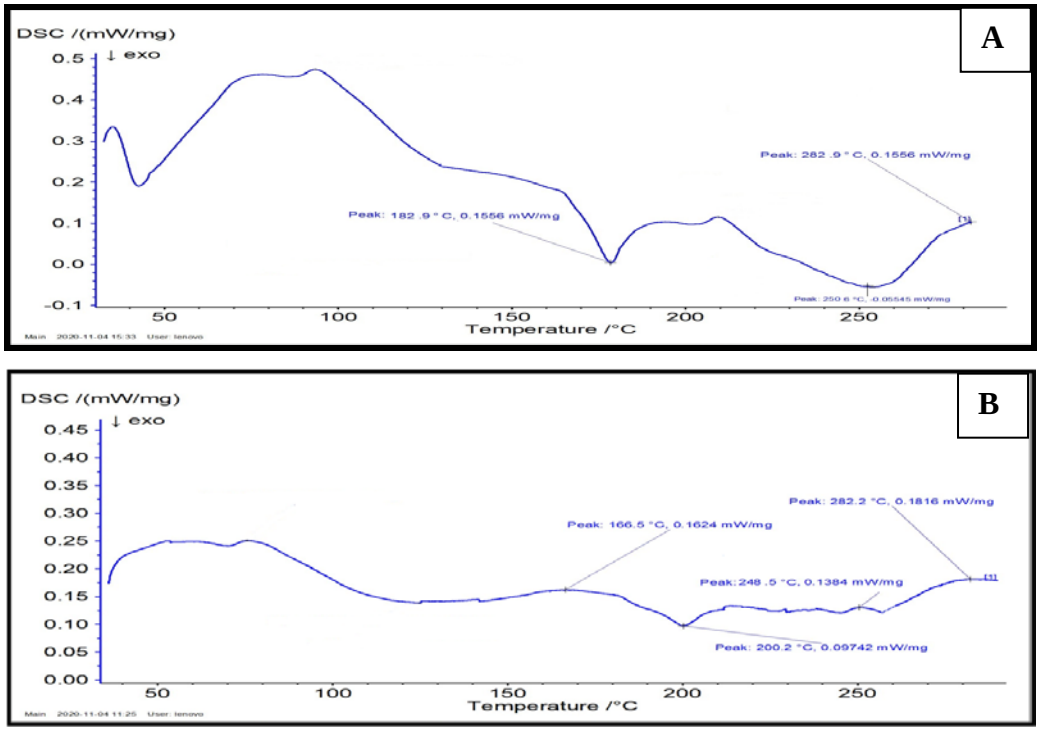


Figure: 4.24 DSC analyses of uncoated (A) and coated (B) pellets.

point. The pectin showed a rise at 182 ºC, which was also nearby to its melting point. From all the peaks, it was confirmed that there was a lack of significant interactions between polymers and drugs, in the case of coated pellets, (Figure 4.24 B) DSC of Mesalamine showed a peak at 282ºC which is relatively closed to its melting point. Similarly, MCC peaked at 248ºC, close to its melting point. The coating material CAP exhibited a peak at 200ºC and pectin at 166ºC, coinciding with their melting points. The observed peaks ensured no significant interaction between the drug and polymers.

4.17 *IN VITRO* DRUG RELEASE

For an efficient colonic drug delivery system, it has to reside unchanged in the upper G.I.T. For plain Mesalamine dissolution, burst drug release was observed. More than 60% of the drug was released in the first 2 hrs in SGF and around 80% of the drug was released within 5 hrs in SIF. In uncoated pellets (MP10), nearly 40% of the drug release was observed within the first 2 hrs in SGF. In contrast, uncoated pellets fail to meet the criteria for preventing premature drug release in SGF and about 62% of the drug was released within 5 hrs and more than 90% of the drug release was observed within 10 hrs. In CAP-coated pellets (MP11), only 4% of the drug was released in the first 2 hrs in SGF. Due to the enteric coating of pellets, CAP can restrict drug release in the stomach and about 13% of the drug release was observed within 5 hrs in SIF. After 10 hrs, more than 55% drug was released and almost the total amount of drug release was accomplished in the 24 hrs in SCF at pH 7.4, as depicted in Figure 4.25. In the case of uncoated pellets, the *in vitro* drug release results showed that around 65% of the drug released was observed within the first 5 hrs. But in CAP-coated pellets, only 13% drug release was observed within the first 5 hrs, indicating that the coating polymer can prevent premature drug release in the stomach and the small intestine. The drug release rate depends on CAP's swelling in the colonic pH. The degree of swelling deteriorates the coating material's pore size and density, influencing the release rate. A significant difference in drug release pattern was observed from coated and uncoated pellets containing mesalamine and probiotics.

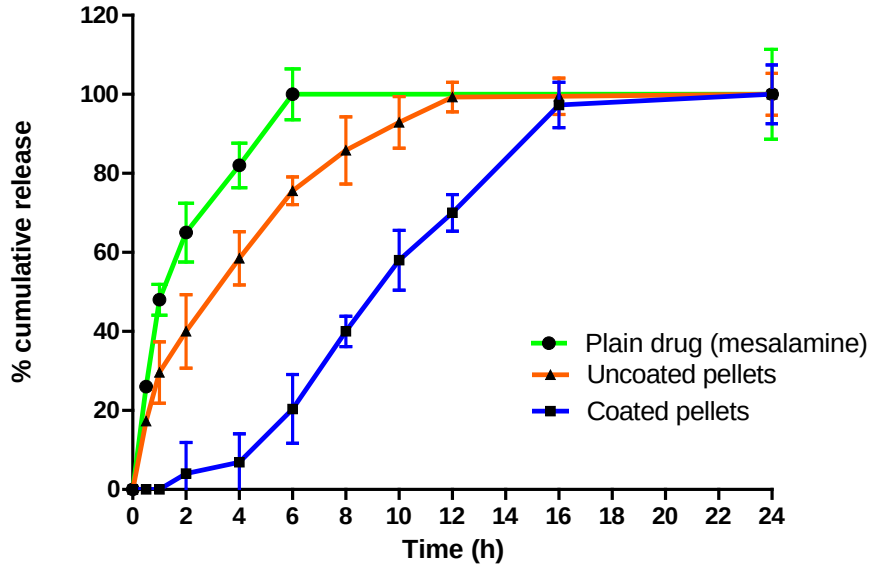


Figure: 4.25 Cumulative % drug release profiles of uncoated and coated pellets in pH 1.2, pH 6.8 and rat cecal content (pH 7.4). Data are shown as mean ± SD.

4.18 *IN VITRO* PROBIOTIC RELEASE AND VIABILITY COUNT

For uncoated formulation, almost 25% of the probiotic was released in 2 hrs in SGF at pH 1.2. More than 70% of the probiotic was released in SIF (pH 6.8) in 5 hrs. Approximately 80% of the probiotic release was observed in 8 hrs at pH of 7.4 SCF. In the case of coated pellets formulation in the first 2 hrs, in SGF at pH 1.2, only 2-4% of the probiotic release was observed, as shown in Figure 4.26. At the 4^{th} hrs pH 6.8, around 20% of the probiotic releases were observed due to the eruption of coating polymer by SIF. But after 6 hrs burst release effect in the probiotic release was observed. The results were expressed as log CFU/g of yeast. A minor rise in viable cell count was noticed by a combination of drug and probiotic in CAP-coated pellets revealing that this formulation might be utilized further for *in vivo* studies. During the 10^{th} hrs in SCF presence, uncoated and coated pellets of *S.boulardii.* showed $2.01x10^{7}$ -$2.07x10^{7}$ CFU/g and, $2.07x10^{7}$ CFU/g - $2.09x10^{7}$ CFU/g viable counts respectively. The *in vitro* release of probiotics was observed to be nearly similar to that of Mesalamine. A slight rise in viable probiotic cell count was observed in coated

pellet release, confirming that the coated pellets could release the probiotic to the specific colonic region.

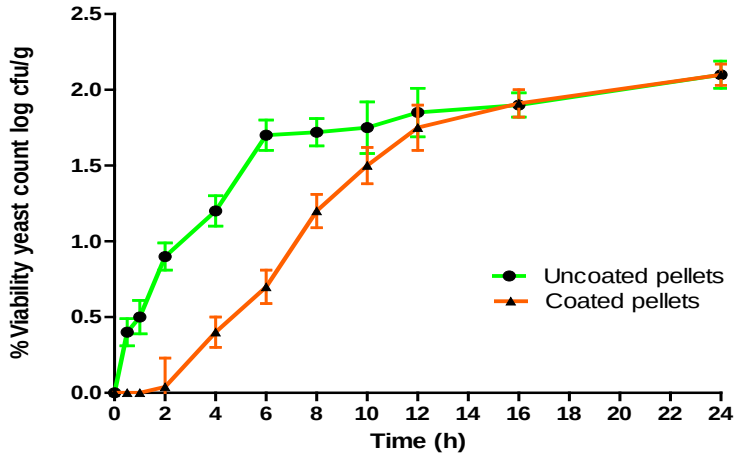


Figure: 4.26 Viability count and release of *S.boulardii* from uncoated pellets and coated pellets in pH 1.2, pH 6.8 and rat pH 7.4. Data are shown as mean ± SD.

4.19 STABILITY STUDY

The stability study was performed at temperatures (25 ± 2 ºC and 40 ± 2 ºC) and (60 ± 5% as well as 75 ± 5%) respectively, with relative humidity for 6 months. The changes in the percentage of drug entrapment, moisture content and percentage cumulative drug release of coated pellets (MP11) formulations have been shown in Table 4.22. There was no significant decrease in percentage drug entrapment, moisture content and percentage cumulative drug release of coated pellets (MP11) formulations indicating high stability of the pellets formulations.

Table: 4.22 Stability of coated pellets loaded with Mesalamine and *S.boulardii* after 6 months

Parameters	**Stability at 25±2 °C**				**Stability at 40±2 °C**			
Coated Pellets	0^{th} day	60^{th} day	120^{th} day	180^{th} day	0^{th} day	60^{th} day	120^{th} day	180^{th} day
% drug entrapment	82.67 ±0.05	81.32±0.04	80.86±0.03	80.21±0.07	82.67±0.05	81.32±0.12	80.73±0.26	78.27±0.17
%Moisture content	4.33±0.4	4.86±0.8	5.29±0.8	5.45±0.6	4.33±0.4	4.21±0.11	3.08±0.15	2.17±0.9
% cumulative drug release	82±6.4	81±4.5	80±1.9	79±2.8	82±6.4	81±3.1	80±3.7	79±2.4

4.20 *IN VIVO* RESULTS

4.20.1 Morphological studies

4.20.1.1 Assessment of body weight

The body weight of Wistar rats exposed to the treatment scheduled for TNBS-induced UC was decreased in contrast to normal control, as shown in Table 4.23. The effects of each treatment schedule were observed in six animal groups on the 0^{th} day, 7^{th} day and last day of the treatment schedule, 15^{th} day. An increase in the body weight of rats was a sign of improvement from the colitis**.**

Table: 4.23 Bodyweight assessments of different animal groups

Group	Treatment	0^{th} Day	7^{th}Day	15^{th}Day
1	Normal control	242.3±4.91	245±7.12	251±6.42
2	Disease control	248.6±6.39	241.5±5.45	233.5±6.67
3	Placebo group	242±4.67	244±5.12	251±5.43
4	Colitis + Mesalamine 23mg/kg pellets	247±5.49	248±4.91	249±7.31
5	Colitis + probiotic (10^9 CFU) pellets	247±8.40	248±7.52	248±6.51
6	Coated pellets containing Mesalamine 23mg/kg + probiotic (10^9 CFU)	253±08.26	256±4.62	262±5.49

4.20.1.2 Macroscopic activity score

The TNBS model of colitis has been used to induce colon inflammation. The percentage change in weight, stool consistency, lesion score, and macroscopic scores for the colitis group was observed to be 4 ± 0.07, 4± 0.04, 4± 0.07 and 4± 0.09 correspondingly. Whereas for CAP-coated microparticles, the scores for the percentage change in weight, stool consistency, score of the lesion and macroscopic

scores for the colitis group were found to be 1± 0.3, 1± 0.08, 1± 0.13, 1± 0.21 correspondingly. The Wistar rats' weight was measured at the beginning and end of the treatment schedule (15^{th} day), as shown in Table 4.24.

Table: 4.24 Evaluation of microscopic parameters for assessment of disease; values are statistically considerable at p<0.05 compared to normal and p<0.05 compared to disease control rats.

Group	Treatment	Scores (%) weight loss	Consistency of Stool Scores	Scores of Lesion	Scores of Macroscopic
1	Normal control	0.0 ± 0	0.0 ± 0	0.0 ± 0	0.0 ± 0
2	Disease control	4 ± 0.21	4± 0.19	4± 0.23	4± 0.31
3	Placebo group	0.0 ± 0	0.0 ± 0	0.0 ± 0	0.0 ± 0
4	Colitis + Mesalamine 23mg/kg pellets	3 ± 0.4	3± 0.9	3± 0.4	3± 0.3
5	Colitis + Probiotic (10^9 CFU) pellets	2± 0.31	2± 0.14	3± 0.23	3± 0.28
6	Coated pellets containing Mesalamine 23mg/kg + probiotic (10^9 CFU)	1± 0.06	1± 0.12	2± 0.13	3± 0.12

4.20.1.3 Diarrhoea assessment during the treatment period

Rank 1 for moderate to mild diarrhea was observed in group I rats for the initial 1-2 days after drug delivery of TNBS. There was a significant change in the stool consistency during the treatment period in rats groups II, IV, V and VI. After administration of coated Mesalamine and probiotic pellets to the rats, a huge

improvement towards normal consistency of fecal and reduced bleeding was observed from the 4th day onwards, as depicted in Figure 4.27. Normal stool consistency (Rank 0) lacking any traces of blood was attained in all the groups at the end of the treatment period.

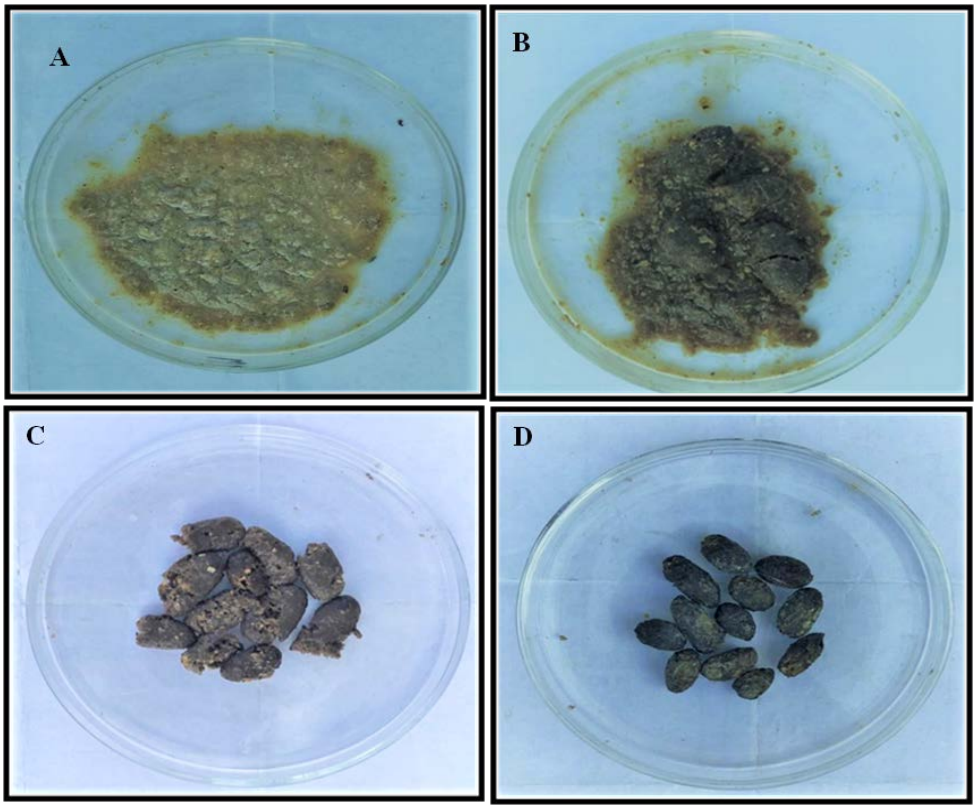


Figure: 4.27 Stool consistencies on different days of treatment A) during 2nd days after induction of UC period, B) After 7th days of the treatment period, C) & D) at the end of the treatment period.

4.20.2 Effect of formulation on MPO in TNBS-induced colitis in Wistar rats

Due to colitis induction, the concentration of colonic enzymes becomes altered. MPO is a colonic enzyme; the activity of this enzyme is concerned with the concentration of neutrophils in the inflamed tissue; therefore, this activity can act as a parameter for the assessment of acute intestinal inflammation. Intrarectal administration of TNBS illustrated a significant rise in MPO concentration of 17.3 μ mol/min/mg tissue, while

the concentration of MPO was almost the same in the disease control group and placebo group. Plain mesalamine pellets reduced MPO concentration to 11.2 µmol/min/mg, near the values obtained after giving plain probiotic pellets, i.e., 11.1 µmol/min/mg. However, CAP-coated pellets of Mesalamine and probiotics revealed a remarkable reduction in MPO concentration, i.e., 8.3 µmol/min/mg, as a contrast to disease control, as shown in Figure 4.28.

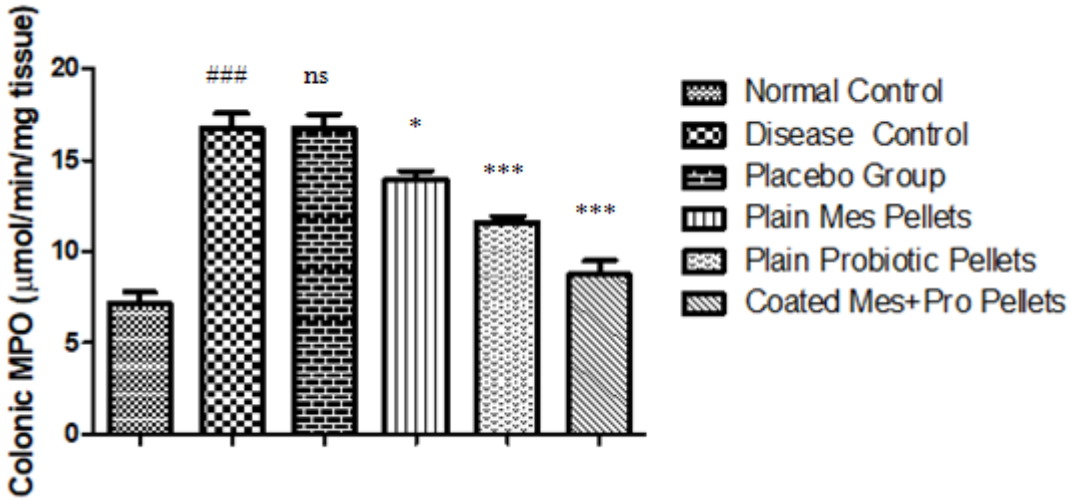


Figure: 4.28 Determination of MPO in colitis rats. Each data represents mean ±S.D. (n=3). Significance was tested using one-way ANOVA and Tukey–Kramer post test. ###p < 0.001 (Normal control vs. disease control group), ^{ns}p > 0.05, *p < 0.05 and ***p < 0.001 (disease control vs. treatment groups).

4.20.3 Effect of formulation on LPO in TNBS-induced colitis in Wistar rats

LPO is a colonic enzyme; the reactive metabolites can be generated due to an increase in the level of LPO, which can lead to inflammation. Intrarectal administration of TNBS showed a significant rise in the LPO concentration in the disease control group, i.e., 150.2 µmol of MDA/mg, while the placebo group had almost the same concentration of LPO in comparison with disease control. The plain Mesalamine pellets reduce the LPO concentration, i.e., 127.3 µmol of MDA/mg. This is near to the

effect of plain probiotic pellets 125.2 µmol of MDA/mg. But CAP- coated Mesalamine pellets with probiotic reduces the LPO concentration to 76.2 µmol of MDA/mg compared to disease control, as shown in Figure 4.29.

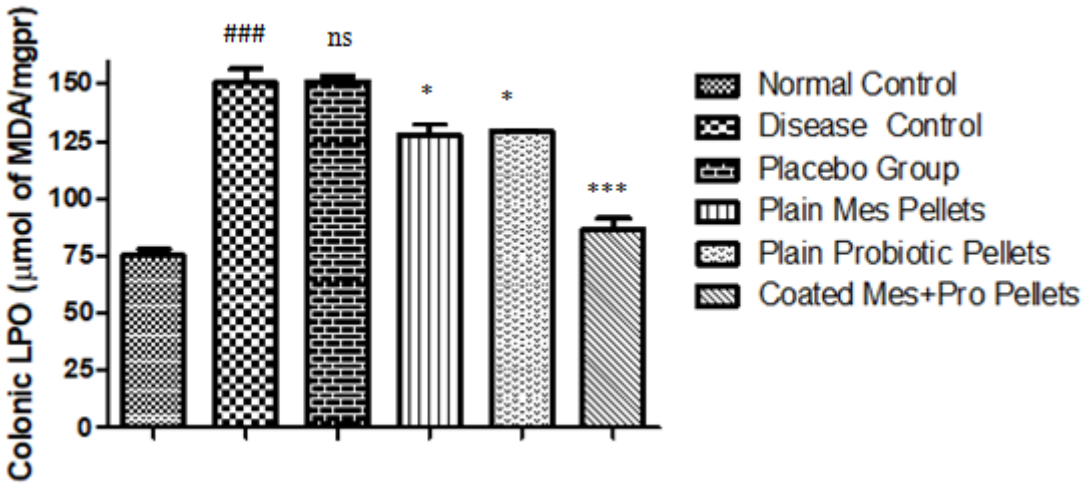


Figure: 4.29 Determination of LPO in colitis rats. Each data represents mean ±S.D. (n =3). Significance was tested using one-way ANOVA and Tukey–Kramer post-test. ###p < 0.001 (Normal control vs. disease control group), ^{ns}p > 0.05, *p < 0.05 and ***p < 0.001 (disease control vs. treatment groups).

4.20.4 Effect of formulation on GSH in TNBS-induced colitis in Wistar rats

GSH is a colonic enzyme involved in DNA repair and is responsible for antioxidant activity. Intrarectal administration of TNBS illustrated a considerable decrease in GSH concentration in the disease control group, i.e., 3.8 µmol of GSH/mgpr, nearly the same as the placebo group value. Plain probiotic pellets gave rise to the GSH concentration, i.e., 4.8 µmol of GSH/mgpr, but plain mesalamine pellets raised the GSH concentration, i.e., 5.1 µmol of GSH/mgpr, which was slightly more significant than the plain probiotic pellets effect. CAP-coated pellets of mesalamine with probiotics showed a significant rise in GSH concentration, i.e., 6.8 µmol of GSH/mgpr as a contrast to disease control, as shown in Figure 4.30. The observed

results have concluded that the CAP-coated pellets formulation containing mesalamine and probiotics is beneficial in maintaining colonic enzymes in UC.

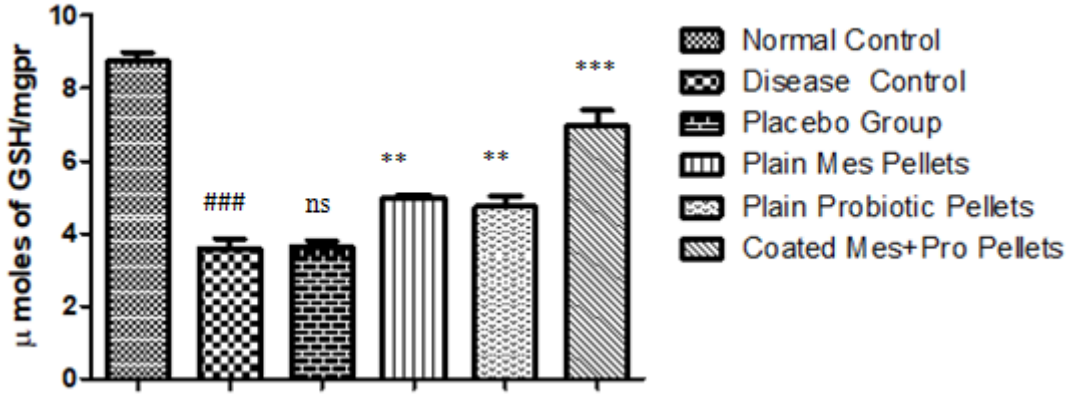


Figure: 4.30 Determination of GSH (Glutathione level) in colitis rats. Each data represents mean ±S.D. (n =3). Significance was tested using one-way ANOVA and Tukey–Kramer post-test. ###p < 0.001 (Normal control vs. disease control group), ^{ns}p > 0.05, **p < 0.01 and ***p < 0.001 (disease control vs. treatment groups).

4.21 DETERMINATION OF C-REACTIVE PROTEIN

CRP is a sensitive marker of inflammation; Table 4.25 showed that CRP levels are increased in UC, but treatment with CAP-coated Mesalamine and probiotic microparticles showed a reduction in CRP levels very effectively. Mesalamine microparticles as well as plain probiotic microparticles. However, the level of CRP was almost similar for disease control and placebo.

4.22 DETERMINATION OF ESR

Erythrocyte sedimentation rate (ESR) is an easy test for assessing inflammatory response. The observed results have shown that CAP-coated microparticles of Mesalamine and probiotics have the property to considerably reduce the ESR compared with plain mesalamine microparticles and plain probiotic microparticles.

However, the ESR level was similar to disease control and the placebo, as shown in Table 4.25.

4.23 DETERMINATION OF WBC

The body releases white blood cells when an infection or inflammatory disease arises to help fight the infection. as shown in Table 4.25, WBC levels are increased after UC due to the cells' proliferation. However, coated microparticles of mesalamine and probiotics have shown a marked reduction in the level of WBCs in comparison with plain microparticles of mesalamine and probiotics, respectively. However, the results are almost identical for the placebo and disease control groups.

Table: 4.25 Determination of WBC, CRP and ESR levels after treatment schedule

S. No.	Group (pellets)	WBC(µl)	CRP (mg/dl)	ESR mm/hr
1	**Group**	10.3 ±0.17× 10^3	2.4±0.27	2.7±0.71
2	Normal control	14.6 ±1.31× 10^3	9.7±0.46	22.2±0.56
3	Disease control	14.4 ±0.56× 10^3	9.89±0.56	20.9±0.98
4	Colitis+ placebo group (Inert material formulation), oral route	13.0±0.83× 10^3	5.3±0.79	15.2±1.14
5	Colitis+Mesalamine pellets (23 mg/kg, oral route)	12.8±1.09 × 10^3	6.2±0.25	19.5±1.74
6	Colitis+probiotic pellets (10^9CFU), oral route	10.9±0.45 × 10^3	3.1±0.42	12.2±0.32

4.24 *IN VIVO* STUDIES

4.24.1 Pharmacokinetics estimation

The concentration against time profiles after oral administration of the uncoated and coated pellets has been presented in Figure 4.29. After uncoated pellets oral

administration, T_{max} was found to be 0.82±0.19, which was significantly distinct ($p<0.05$) from the 5.91±0.77 T_{max} obtained from the coated pellets. The uncoated pellets' observed C_{max} (3.15±0.98 mg/ml) was much higher than that obtained from the coated pellets (1.06±0.37 mg/ml). The determined pharmacokinetic parameters have been given in Table 4.26. The MRT value of 8.36 hrs of the drug obtained from the uncoated pellets was much lower than that of the coated pellets (16.23 hrs). The AUC from the uncoated pellets (14.05±1.53 µg/ml/h) was lower than that obtained from the coated pellets (13.02±1.31µg/ml/h). The possible reason for this may be the hydrophilic nature of the mesalamine, which results in poor uptake from the colon to the blood, suggesting that a minimal amount of the drug was able to diffuse into the plasma through the lipid membrane. Pharmacokinetic outcome found the controlled drug release manners of the optimized pellet formulation, which recommends more efficient colitis management by supplying elevated high drug concentration at the colonic site. The pharmacokinetic results confirmed that the C_{max} of uncoated pellets was 3.15±0.98 (mg/ml) and 1.06±0.37 (mg/ml) for coated pellets. This was comparatively more than that of CAP-coated, indicating less. The amount of drug that enters the systemic circulation in the case of CAP-coated pellets formulation

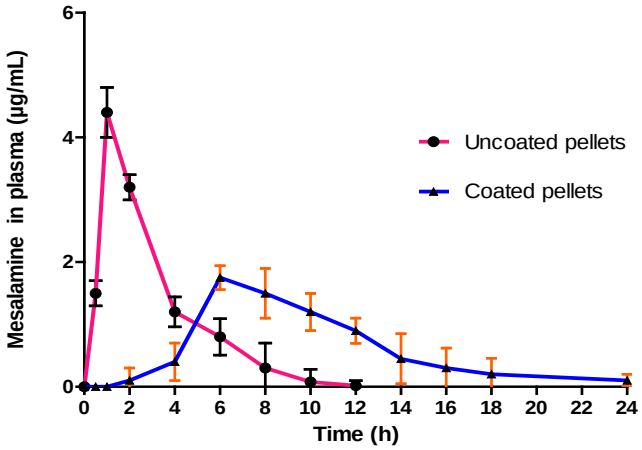


Figure: 4.31 Plasma concentration profiles of Mesalamine after oral administration of uncoated and coated pellets in Wistar rats. Data are shown as mean±SD (n=3).

Table: 4.26 Pharmacokinetic parameters of Mesalamine after oral administration of uncoated and coated pellets

Parameters	Uncoated Pellets	Coated Pellets
C_{max} (mg/ml)	3.15±0.98	1.06±0.37
T_{max} (h)	0.82±0.19	5.91±0.77
AUC_{total} (µg//ml/h)	14.05±1.53	13.02±1.31
$T_{1/2}$	5.45±0.21	11.25±1.45
MRT (h)	8.36±0.67	16.23±2.23

4.25 HISTOPATHOLOGICAL ANALYSIS

In the histopathology, the colon tissue of group I was observed as dark pink with no macroscopic damage. The colonic specimen of group II mucosal injury was indicated by the deficiency of epithelial cells and increased mucosal/submucosal infiltration of inflammatory cells. The colonic section of group III mucosal injury was confirmed by the lack of epithelium cells and the occurrence of a substantial mucosal/submucosal infiltration of inflammatory cells, same as in disease control with increased lamina propia with depletion and distribution of mucosal gland feature. The colonic tissue of group IV revealed little distortion of the mucosal layer and disturbances in epithelium layers at few places. Inflammation with moderate inflammatory cells and infiltration was also seen. The regeneration of crypts was also examined in various places. In group V after treatment with plain mesalamine pellets, continuous mucosa erosion with some inflammation signs and a slight change has been observed. The colonic specimen of group VI revealed a remarkable difference after administering CAP-coated pellets of mesalamine and probiotics via the oral route. From the histopathological results, the CAP-coated pellets of Mesalamine and probiotic showed marked restoration of goblet cells, cytoplasmic mucin and crypt with slight inflammation. The overall appearance was similar to normal colon histopathology, as depicted in Figure 4.32.

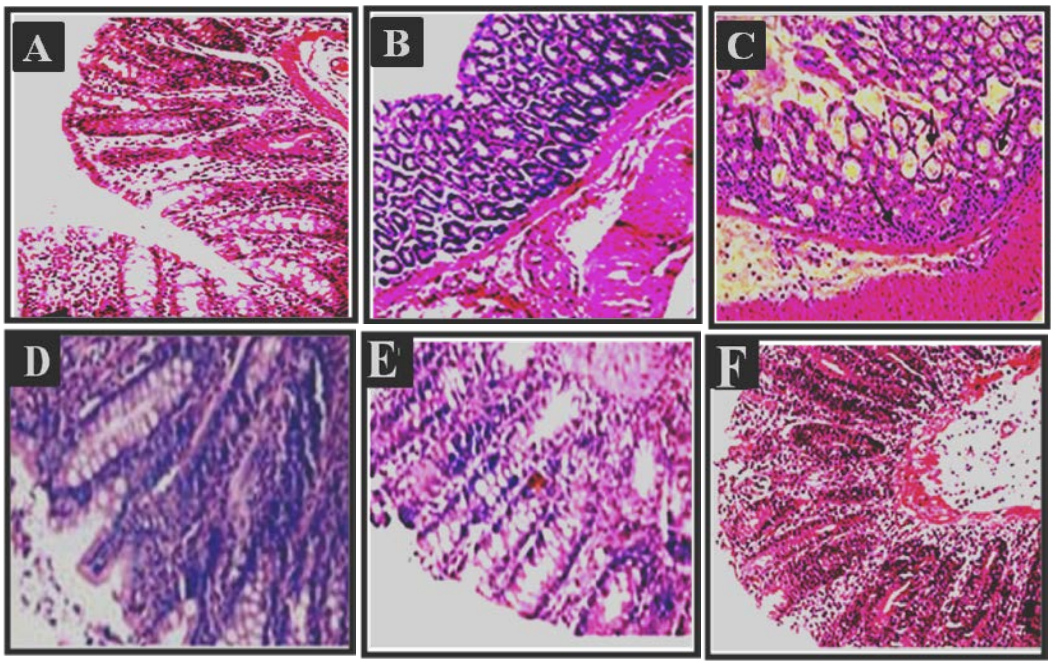


Figure: 4.32 Histopathological change in colon experimental of Wistar rat after pellets formulation; A- Normal control, B- Disease control, C- Placebo group, D-Plain Mesalamine pellets (23 mg/kg, oral route), E-Plain probiotic pellets (10^9 CFU), F- Coated pellets of Mesalamine +probiotic (10^9 CFU), through oral administration.