5. DISCUSSION

The objective of the work was to utilize the colon specific drug delivery for the treatment of inflammatory bowel disease to achieve better therapeutic effects and lesser side effects. Colon targeted drug delivery systems for curcumin and flurbiprofen were developed to bypass the upper GIT and specific release at the colonic pH. Curcumin (CUR) and Flurbiprofen (FLB) microspheres were designed with the objective of controlled and targeted release of drug to colon. Drug delivery to colon encounters obstacle such as absorption and degradation in the upper GIT. The optimum drug delivery to colon requires avoidance of the absorption and metabolic breakdown of these drugs from the stomach and small intestine. Therefore, pH sensitive polymer Eudragit S 100 was chosen which can bypasses the upper GIT and has the ability to retain on the surface of colon ensures local and targeted effect.

Curcumin can chelate various metal ions to form metallocomplexes of curcumin, which can show greater effects than curcumin alone. In this work the curcumin complex with zinc metal was also studied for its potential anti-inflammatory activity in experimentally induced ulcerative colitis. Curcumin may confer some additional therapeutic advantages when used in combination with certain conventional anti-inflammatory drugs. Curcumin complex along with Flurbiprofen was also studied for its synergistic effect in ulcerative colitis.

5.1. Preformulation studies of Curcumin

The gift sample of curcumin was identified by various organoleptic, physicochemical and spectroscopic methods. The sample of curcumin possessed similar color, odor and texture as given in officials. The melting point of obtained sample was found to be 182°C ± 1.28 which was within the range as reported in the literature (Aggarwal et al., 2006) confirmed identity of the drug. Thin Layer Chromatography was also performed and the solvent system used was chloroform: methanol (9:1). The Rf value of the curcumin was found to be 0.92 ± 0.015 which was almost same as reported in the literature (Vedamurthy et al., 2010), confirmed the identity of the drug. The identification of the drug was further confirmed by its FT-IR spectroscopy in which the spectrum was analyzed to confirm the presence of various functional groups present in curcumin. The major peaks of functional groups and the finger print region of sample drug were found to be same as that of reference (Vedamurthy et al., 2010) again confirmed the authenticity of sample drug.
The calibration curves of Curcumin were prepared in 0.1 N HCl (pH 1.2), phosphate buffer (pH 6.8) and in phosphate buffer (pH 7.4). The graph between different concentrations of curcumin and absorbance were found to be linear in the concentration range of 5-30 µg/mL with a regression coefficient of 0.999 (phosphate buffer pH 7.4), 0.9995 (phosphate buffer pH 6.8), 0.9996 (0.1N HCl pH 1.2) at 432 nm using UV spectrophotometer (Systronics, 2202). Regarding solubility, it was found that solubility of curcumin was more in phosphate buffer pH 7.4 (2.68±0.041) as compared to phosphate buffer pH 6.8 (0.83 ± 0.013) than 0.1N HCl pH 1.2 (0.66 ± 0.09). DSC study revealed the compatibility between drug and excipients.

5.2. In-vitro evaluation of CUR microspheres

Chitosan microspheres and Eudragit coated chitosan microspheres of curcumin were prepared by the emulsion cross linking method. These prepared microspheres were also evaluated for their various quality control parameters. The formulated microspheres were examined for particle size, shape, surface morphology, percentage yield, drug loading, entrapment efficiency (EE) and degree of swelling.

SEM photomicrograph of chitosan microspheres indicated that the cross-linked chitosan microspheres exhibited rough surface and spherical shape while SEM photomicrograph of Eudragit coated chitosan microspheres revealed smooth and spherical. The size of microspheres was found to increase (36.84µm to 77.25µm) with increase in chitosan concentration. Further the coating with Eudragit also showed significant increase in the size of microspheres. The mean particle size of the coated microspheres increased from 93.27µm to 129.74µm, which may be due to the corresponding increase in the chitosan concentration that resulted in larger emulsion droplets.

X-ray diffractogram revealed that after the entrapment of drug into chitosan polymer the intensity of peaks markedly decreased. This suggested inclusion of CUR into microsphere formulation. Analysis of the diffractograms revealed higher crystallinity of the CUR as compared with the microspheres.

The EE was found to increase with increase in drug-polymer ratio and F4 formulation exhibited the highest EE consisting drug-polymer ratio of 1:4. As the concentration of chitosan increased, degree of swelling also increased due to the swelling property of chitosan polymer. Higher concentration of chitosan resulted in higher uptake of phosphate buffer (pH 7.4), which resulted
in higher degree of swelling. Eudragit S100 coating tends to dissolve in phosphate buffer and chitosan microspheres exposed to the phosphate buffer.

The prepared microspheres were also evaluated for In-vitro drug release. The pH conditions of the dissolution studies were selected so as to simulate the GI conditions. In-vitro drug release of CUR from the chitosan microspheres revealed 15.97±0.64% to 20.25±0.87 % of drug was released in the initial 4h indicating burst release of drug. These types of dissolution profiles are not appropriate for colon targeted drug delivery system. For an effective delivery of drug specific to colon, the drug release should be kept minimum while its transit through the upper GIT so as to ensure the maximum dose of drug reaches to colon. Burst release of CUR from chitosan microspheres might be due to solubilization of chitosan at acidic pH. This problem was overcome by coating of chitosan microspheres with Eudragit S-100 as it exhibit pH dependent solubility with threshold pH of 7.0. The Eudragit coating of chitosan microspheres was attained by using emulsification solvent evaporation method. Eudragit coating of chitosan microspheres prevented the premature release of CUR in the upper GIT. The release rate of CUR from Eudragit coated microspheres was found to increase after 4 h, due to the exposure of microspheres to pH 7.4, which is more than the solubilizing pH of Eudragit S-100 polymer. In the presence of pepsin, Eudragit S-100 coating was found to be protected and exhibited unchanged in-vitro release profile up to 4 h. The mechanism of release from the developed microspheres was supposed to follow the following pattern: a) dissolution of Eudragit coating above pH 7.0, b) swelling of chitosan and c) diffusion of CUR from swollen chitosan gel. The release rate of drug from the microspheres was also affected by the chitosan concentration as with the increase in the concentration of chitosan, the release rate was found to decrease may be due to the fact that drug has to travel more path length in case of higher concentration of chitosan. For colon targeted drug delivery the conventional dissolution study is not able to predict accurately the in-vivo performance of targeted system for which the release of drug is triggered by colonic microflora. Therefore the release study of developed formulation was carried out in the presence of rat caecal contents from the 5th hour in the simulated colonic fluid. The release of CUR was increased significantly in presence of rat caecal contents, which suggested biodegradability of chitosan in the presence of colonic microflora. A drug release of 46.95±0.88 % (F6) was found with rat caecal contents after 12 h while it was 37.53±0.58 % in absence of rat caecal contents.
On the basis of best correlation co-efficient the *in-vitro* release data seem to fit better with Higuchi model, thus suggested diffusion as the main mechanism for drug release. Among the various Eudragit coated microsphere formulations the F6 exhibited best pattern of release with highest value of regression coefficient $r^2$ (0.9404) and the release exponent of Peppas model revealed super case II diffusion kinetics.

### 5.3. Characterization of Curcumin-Zn(II) complex

Curcumin-Zn(II) complex was successfully prepared and characterized by TLC, IR, UV, $^1$HNMR. The results of TLC (Rf value) of Curcumin-Zn(II) complex (0.81 ± 0.05) as compare to curcumin alone (0.92 ± 0.015) revealed formation of complex. In IR Spectroscopy, shifting of stretching vibrations of $\nu$(C=C) and $\nu$(C=O) in Curcumin-Zn(II) complex compared to CUR confirmed the formation of metallocomplex. UV spectra are deconvoluted with absorption band at 432nm for CUR and at 466.4nm for Curcumin-Zn(II) revealed the formation of complex. $^1$HNMR spectra of Curcumin-Zn(II) clearly showed the upfield shift of $H_a$ and $H_b$ due to diamagnetic isotropic effect because of the presence of zinc ion in close vicinity suggested the presence of Zn complex.

The calibration curve of Curcumin-Zn(II) were prepared in 0.1N HCl (pH 1.2), Phosphate buffer (pH 6.8) and Phosphate buffer (pH 7.4). The graph between different concentrations of Curcumin-Zn(II) and absorbance were found to be linear in the concentration range of 5-30 $\mu$g/mL with a regression coefficient of 0.999 (phosphate buffer pH 7.4), 0.9997 (phosphate buffer pH 6.8) and 0.9991 (0.1N HCl pH 1.2) at 466 nm using UV spectrophotometer (Systronics, 2202).

Since curcumin has a preferred enolate structure, it appears to be very ideal for making chelated complexes with di & trivalent inorganic molecules like Zn$^{2+}$, Al$^{3+}$, Fe$^{3+}$ since curcumin has C=O & enolate, hydroxyl in 1,3 positions. Thus making curcumin as a stable complex with ZnCl$_2$ and making it more soluble for transportation by blood stream.

### 5.4. In-vitro and in-vivo evaluation of Curcumin-Zn(II) microspheres

Chitosan microspheres and Eudragit coated microspheres of Curcumin-Zn(II) were successfully prepared by emulsification cross linking method. The developed microspheres were subjected to various evaluation parameters like particle size, particle shape and surface morphology.
SEM photomicrograph of Eudragit coated chitosan microspheres showed smooth and spherical shape. With the increase in concentration of chitosan, the mean diameter of microspheres also increased and Eudragit coated chitosan microspheres further increased the size of microspheres (101.23μm to 125.97μm) which may be due to increase in the concentration of chitosan that leads to larger emulsion droplets.

X-ray diffractogram resulted in decreased peak intensity of microspheres of Curcumin-Zn(II) in comparison with free Curcumin-Zn(II), suggested inclusion of drug within microsphere formulation.

Prepared microspheres were further examined for entrapment efficiency, in-vitro swelling and in-vitro drug release. The EE was found to increase with increase in drug-polymer ratio. In-vitro swelling increased with increase in chitosan concentration due to the swelling property of chitosan polymer as high amount of chitosan would absorb the higher amount of phosphate buffer. Swelling of microspheres is desirable for the diffusion of drug through the microspheres.

In-vitro drug release of Curcumin-Zn(II) from the chitosan microspheres in the initial 4h resulted 25.79±0.87% to 40.65±1.22% of drug indicating burst release of drug. These types of dissolution profiles are not appropriate for colon targeted drug delivery system. Burst release of Curcumin-Zn(II) from chitosan microspheres might be due to solubilization of chitosan at acidic pH. Eudragit S-100 is a pH dependent polymer having threshold pH 7.0. Eudragit coating of chitosan microspheres prevented the premature release of Curcumin-Zn(II) in the upper GIT. The release rate of Curcumin-Zn(II) from Eudragit coated microspheres was found to increase after 4 h, due to the exposure of microspheres to pH 7.4, which is more than the solubilizing pH of Eudragit S-100 polymer. In the presence of pepsin, Eudragit S-100 coating was found to be protected and exhibited unchanged in-vitro release profile up to 4 h. The mechanism of release from the developed microspheres was supposed to be same as stated in curcumin microspheres. The mechanism of release from the developed microspheres was supposed to follow the same pattern as that of curcumin. The drug release from the microspheres was also affected by chitosan concentration as with the increase in the concentration of chitosan, the release rate was found to decrease may be due to the fact that drug has to travel more path length in case of higher concentration of chitosan. For colon targeted drug delivery the conventional dissolution study is not able to predict accurately the in-vivo performance of targeted system for which the release of drug is triggered by colonic microflora. Therefore the release study of developed formulation
was carried out in the presence of rat caecal contents from the 5th hour in the simulated colonic fluid. The release of Curcumin-Zn(II) was increased significantly in presence of rat caecal contents, which suggested biodegradability of chitosan in the presence of colonic microflora. A drug release of 84.16±1.12% was found with rat caecal contents after 12 h while it was 77.29±0.94 % in absence of rat caecal contents.

On the basis of best correlation co-efficient the in-vitro release data seem to fit better with Higuchi model, thus suggested diffusion as the main mechanism for drug release. Among the various Eudragit coated microsphere formulations the F14 exhibited best pattern of release with highest value of $r^2$ (0.9963) and the release exponent of Peppas model revealed super case II diffusion kinetics.

In-vivo organ distribution study of optimized formulation (F14) was carried out in order to analyze its targeting potential in the colon and results suggested protection of microspheres in upper GIT and the drug was released after reaching to colon due to solubilization of Eudragit coating and microbial degradation of chitosan.

In-vivo study using acetic acid induced colitis model in control group showed that the caecum, colon and rectum had evidence of mucosal erosion, congestion and hemorrhagic ulceration. The treatment with pure CUR showed insignificant reduction in severity and extent of the colonic damage as compared to control group while significant reduction in severity and extent of colonic damage was observed with Curcumin-Zn(II) loaded microspheres. The elevated weight to length ratio of colon was reduced by Curcumin-Zn(II) loaded microspheres indicating its healing property. The results were also supported by histopathological studies, which showed that extensive necrosis of colonic epithelium and hemorrhagic lesions of the entire colonic mucosa in acetic acid treated control group. The CUR treated group showed negligible reduction in the severity of colitis whereas these disturbances were healed significantly in Curcumin-Zn(II) loaded microsphere treated group.

### 5.5. Preformulation studies of Flurbiprofen (FLB)

The gift sample of FLB was identified by various techniques such as melting point determination, thin layer chromatography, IR spectroscopy etc. The melting point of obtained sample was found to be 110 °C ± 1.15 which was within the range as reported in the literature (The Merck Index, 2006) confirmed identity of the drug. In Thin Layer Chromatography, Rf
value of the FLB using solvent system chloroform: acetone (4:1) was found to be $0.314 \pm 0.027$ which was very closed to reported value (Nair et al., 2013), confirmed identity of the drug. The identification of the drug was further confirmed by its FT-IR spectroscopy in which the spectrum was analyzed to confirm the presence of various functional groups along with the finger print region present in FLB. The major peaks of functional groups of sample drug were found to be same as that of reference [JP XV] again confirmed the authenticity of sample drug.

The calibration curves of FLB were prepared in 0.1 N HCl (pH 1.2), phosphate buffer (pH 6.8) and in phosphate buffer (pH 7.4). The graph between different concentrations of FLB and absorbance were found to be linear in the concentration range of 5-30 µg/ml with a regression coefficient of 0.9993 (phosphate buffer pH 7.4), 0.9996 (phosphate buffer pH 6.8) and 0.9997 (0.1N HCl pH 1.2) at 254 nm using UV spectrophotometer (Systronics, 2202). Solubility of FLB was found to be more in phosphate buffer pH 7.4 (12.36 ± 0.51) as compared to phosphate buffer pH 6.8 (8.36 ± 0.27) than 0.1N HCl pH 1.2 (6.6 ± 0.036). DSC study revealed the compatibility between drug and excipients.

**5.6. In-vitro and in-vivo evaluation of FLB microspheres**

Microspheres of FLB were prepared by emulsification cross linking method and the developed microspheres were characterized for same evaluation parameters as stated above. Shape and surface morphology were characterized using scanning electron microscopy. Chitosan microspheres possessed a nearly smooth surface and spherical shape. The size of the particle ($Z_{avg}$) increased with increasing concentration of polymer (1:1 to 1:4). Eudragit coating further showed significant increase in the size of microspheres which may be due to formation of larger emulsion droplets because of increase in polymer concentration.

X-ray diffractogram of FLB resulted in several peaks of different intensities while FLB- chitosan microspheres spectra exhibited low intense peaks in the same range revealed inclusion of FLB into microsphere formulation. Analysis of the diffractograms seems to indicate a higher crystallinity of the drug in comparison with the microspheres.

The EE increased with increasing drug–polymer ratio, and the highest value was observed for F20 which employed a drug–polymer ratio of 1:4. Other formulations showed EE values between 79.23% and 87.91%. The degree of swelling was also increased with increase in concentration of polymer due to swelling property of chitosan polymer.
In-vitro release results showed that 37.23–60.89% of drug was released from the FLB-chitosan microspheres in the initial 4h while Eudragit coated chitosan microspheres showed only 6.79-10.01% of drug release after 4 h. The drug release studies suggested that burst release of FLB occurs from chitosan microspheres due to solubilization of chitosan in acidic pH. Eudragit coating of chitosan microspheres offered a high degree of protection from the burst release of FLB in the upper GIT, since Eudragit is pH sensitive polymer having threshold pH of 7.0. FLB release rate from Eudragit coated microspheres increased after 4 h, due to the exposure of formulations to pH 7.4, which is above the solubilizing pH of Eudragit S-100 polymer. Further the release rate of drug from the formulation was also depends upon the concentration of chitosan used. The release rate decreased with increase in the concentration of chitosan. The release of FLB from chitosan microspheres is expected to occur due to swelling of the polymer, resulting in the formation of a gel and followed by diffusion of drug through the gel. So in case of high concentration of chitosan the drug have to travel more path length. The release studies of F23 formulation was also carried out in presence of pepsin and rat caecal contents to mimic the GIT environment. Eudragit S-100 coating was found to be unaffected in the presence of pepsin and showed unchanged drug release profile upto 4 h. In presence of rat caecal contents, the drug release was found to increase significantly, which indicated the biodegradability of chitosan in presence of colonic microflora. A drug release of 92.78% was observed after 12 h in presence of rat caecal content where as it was 84.23% without rat caecal content.

On the basis of correlation co-efficient the release data seem to better fit with Higuchi model, thus suggested diffusion as the mechanism for drug release. Among the Eudragit coated FLB-chitosan microsphere formulations the F23 showed the best release pattern with highest r² value of 0.9908. Further the release data was fitted for release exponent of Peppas model which confirmed that the formulations showed super case II diffusion kinetics.

In order to analyze its targeting potential in the colon, in-vivo organ distribution study of optimized formulation (F23) was carried out and results suggested protection of microspheres in upper GIT and the drug was released after reaching to colon due to solubilization of Eudragit coating and microbial degradation of chitosan.

In-vivo study using acetic acid induced colitis model in control group showed that the caecum, colon and rectum had evidence of mucosal erosion, congestion and hemorrhagic ulceration. The treatment with pure FLB showed insignificant reduction in severity and extent of the colonic...
damage as compared to control group while significant reduction in severity and extent of colonic damage was observed with FLB loaded microspheres. The elevated weight to length ratio of colon was reduced by FLB loaded microspheres indicating its healing property. The results were also supported by histopathological studies, which showed that extensive necrosis of colonic epithelium and hemorrhagic lesions of the entire colonic mucosa in acetic acid treated control group. The FLB treated group showed negligible reduction in the severity of colitis whereas these disturbances were healed significantly in FLB loaded microsphere treated group.

The stability studies of optimized formulation (F14 and F23) were carried in accordance to ICH Q1 A guidelines for 3 months to investigate the influence of humidity and temperature on appearance and in-vitro release of drugs. Results showed that formulation was stable when store in sealed as well as unsealed containers at 40°C ± 2.0 / 75% RH ± 5 in terms of appearance and color, which remained unchanged and had comparable in-vitro drug release, thus suggesting that there was no problem of stability of F14 and F23.

Microspheres of Curcumin-Zn(II) (F14) [10 mg/kg equivalent of Curcumin-Zn(II)] and Flurbiprofen (F23) [2.5 mg/kg equivalent of FLB] in combination were also analyzed by acetic acid induced ulcerative colitis in mice in order to analyze the synergistic effect of combinational formulations.

5.7. In-vivo study of microspheres of Curcumin-Zn(II) and Flurbiprofen in combination
The in-vivo treatment with combination of microspheres (F14 and F23) resulted in 79.96% inhibition in extent and severity of the colonic damage compared to control, whereas treatment with FLB- microspheres (F23) resulted in 67.95% and treatment with Curcumin-Zn(II) microspheres (F14) resulted in 47.92 % inhibition in the extent and severity of the colonic damage. The results were also supported by histopathological studies, which showed negligible signs of necrosis and tissue damage after treatment with combination of F14 and F23. No hemorrhagic or red spots were found on the stomach walls of control animals as well as animals treated with combinations of microspheres. Oral acute toxicity study of combination of F14 and F23 in mice was carried out as per OECD guideline 423 and it was found that that there is no reduction in the alertness, spontaneous motor activity, reactivity to sound and touch, body and limb tone. Respiration, urination, pupil size, reflexes (pinnal, corneal, righting) were found normal for all 14 days of study. Abnormal signs pertaining to toxicity such as ataxia, body
tremors, convulsions, lacrimation, salivation, diarrhoea, writhing, sedation, coma, cyanosis etc., were not observed in all mice during experimental tenure of 14 days.