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Preparation and Investigation of Cytotoxic Activity of Meloxicam Loaded Chitosan Nanoparticles in HT29 Colon Cancer Cell Lines

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Original Research Article

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Abstract: Meloxicam is non-steroidal anti-inflammatory agent and having multiple pharmacological activities have been reported. In the present article involve the development of chitosan nanoparticles of meloxicam and have been subjected to stability test at various pH, drug release study, in vivo pharmacokinetics in wistar rats, and investigated the decreasing the growth of colon cancer cells. Chitosan nanoparticles of meloxicam such as F1 and F2 have shown anticancer activity against HT29 cell lines. Furthermore,. The nanoparticles were in the size range between 170 nm and 220 nm. The oral absorption of nanoparticles were quite higher than the meloxicam alone. Therefore the prepared nanoparticles formulations were showed better activity than meloxicam alone.
Keywords: Meloxicam, Chitosan, Nanoparticles, Cytotoxicity, Dug stability.

INTRODUCTION

Meloxicam (MX) is non-steroidal anti-inflammatory drug which is used in treatment of rheumatoid arthritis and osteoarthritis and has half-life of 20h [1]. MX has superior *in vitro* and in *vivo* inhibitory action against enzyme cyclo-oxygenase (COX-2), which is responsible for inflammation [2]. MX has high potential in the inhibition of adjuvant arthritis several times higher than indomethacin, piroxicam, naproxen and Diclofenac[3].Besides its anti-inflammatory activities, MX has been exploited for various therapeutics benefits like inhibition of growth of colorectal cancer[4] antinociceptive activity[5] potential prophylactic in colorectal cancer[6]antiproliferative effect in prostate cancer[7] prevents tumour growth in murine model[8] treatment of urinary bladder cancer [9] and esophagus squamous cell carcinoma[10].

Literature survey revealed that various formulations of MX has been reported such as MX-cyclodextrin binary systems[11] gel formulations of MX [12]suspension of MX [13] and nanosponges of MX[14]. The aim of the present study is to formulate chitosan nanoparticles, to evaluate pharmacokinetic parameters and to determine the cytotoxic activity in HT29 cell lines. MX is effective in the irreversible inhibition of COX-2 which is one of the enzymes implicated in the development of cancer and MX has been proved to be safest in the management of various cancers and having least unwanted effects compared to specific anticancer or chemotherapeutic agents. To maximize the benefits of anticancer activity and to improve the absorption profile of MX, we have formulated chitosan nanoparticles to study cytotoxicity against colon cancer cell lines. Nanoparticles offer improved biodistribution, increases circulation, promotes enhanced permeability and drug retention [15].Chitosan is a bio-degradable, bio-compatible and non-toxic polymer with mucoadhesive properties [16]. Chitosan nanoparticles have greater drug loading capabilities and able to show sustained drug release properties[17].Chitosan nanoparticles are prepared by ionotropic gelation method which does involve interaction between the negative groups of sodium tripolyphosphate and the positively charged amino groups of chitosan[18].Furthermore, drug loading, drug stability, oral pharmacokinetics and anticancer activity in cell lines were studies.

MATERIALS AND METHODS

Chitosan was purchased from Sigma Aldrich, USA. Sodium tripolyphosphate was purchased from Central Drug House, Delhi, India. Water purified by reverse osmosis, MilliQ, USA and further filtered by 0.22 µm membrane filter. HPLC grade acetonitrile was purchased from SD FineChem, India. Lutrol F 127 was purchased from Himedia, India. Potassium dihydrogen phosphate and all other chemicals were used of analytical grade.

HPLC Analytical method

To determine the concentration of MX in various polymeric and biological matrices, valid analytical method was utilized. Accurately weighed quantity (10 mg) of MX was transferred to 10 ml volumetric flask and further concentrations (1, 3, 5 and 10 µg/ml) were prepared using acetonitrile:water (65:35 v/v) and injected into Shimadzu Binary Prominence HPLC. The chromatographic conditions include 250 mm x 4.6 mm C-18 column having 5 µm, mobile phase acetonitrile:water (65:35 v/v), flow rate 1 ml/min and detected at wavelength of 320 nm. The calibration curve was plotted concentration vs. area and the linearity was established. The linear equation was found to be Area= 68214x concentration + 16134, retention time was 2.35 min and correlation coefficient was found to be 0.999 with relative standard deviation is less than 1% indicates the precision of method.

Preparation of chitosan nanoparticles

Chitosan nanoparticles were prepared by ionic cross linking of chitosan solution with sodium tripolyphosphate prepared in the presence of Lutrol F 127 as a suspending agent to avoid aggregation, at 25° C under continuous stirring. Chitosan nanoparticles were prepared by dispersing 10 mg of MX in 10 ml of chitosan solution (2 mg/ml and 4 mg/ml) containing 0.5% Lutrol F 127 and further added tripolyphosphate (0.2% w/v). The tubes containing nanoparticles were centrifuged at 10000 x g for 45 min using cooling centrifuge and supernatant was discarded.

Characterization of the nanoparticles

The particle size of the MX formulations and polydispersity index were determined using dynamic light scattering equipment (Zetasizer ZS 90). The polydispersity index has a value between 0 and 1. The value close to zero represents particle distribution is narrow and having minimum particle size.

Determination of MX loading capacity and efficiency

Loading efficiency and loading capacity of nanoparticles of MX were evaluated by taking the samples at 10000 x g at -5° C for 45 min. The amount of free MX was present in the clear supernatant after centrifugation was determined by using HPLC validated analytical method. The loading capacity and loading efficiency of MX in nanoparticles were determined from the following equations 1 and 2 mentioned below:

Loading capacity = ((P-Q)×100) x R) Equation (1)
Loading efficiency = ((P-Q)×100 x R) Equation (2)

Where, P is the total amount of MX, Q is the free amount of MX, R is the weight of nanoparticles.

Stability study of MX and nanoparticles at different pH

To determine the stability at various pH, 0.1 M hydrochloric acid, phosphate buffers of pH 3.5, 5.5, 6.8 and 7.4 were selected. Accurately weighed 10 mg of MX and MX loaded nanoparticles were transferred to 2 ml centrifuge tube and 1 ml of each buffer was added to tubes containing nanoparticles and incubated at 25° C for 24 h. The drug content was determined using HPLC method and the extent of drug degradation was evaluated.

In vitro release of MX from nanoparticles

Aliquots of 1 ml MX loaded nanoparticle formulations were centrifuged at 10000 x g. The pellet obtained after decanting the supernatant was diluted with 1 ml phosphate buffered saline (pH 7.4) and incubated at 37° C under shaking equipment (50 rpm) for 3 h. At various time intervals, a tube was selected and centrifuged at 10000 x g for 15 min. The released MX was determined using validated analytical method.

Determination of bioavailability of MX loaded chitosan nanoparticles

Bioavailability is the rate and extent of drug absorption into systemic circulation. To elicit any pharmacological activity, the maximum amount of drug should reach systemic circulation. For these reasons, MX alone and nanoparticles have been administered orally to wistar rats to determine the amount of MX reaching systemic circulation. Vageswari College of Pharmacy, Karimnagar has approved animal facility with CPCSEA registration No. 1720/po/a/13/cpcsea and protocol was approved by institutional Animal Ethics Commitee (IAEC). MX and MX loaded nanoparticles were taken equivalent to 5 mg of MX and administered to male wistar rats (~280 g) orally and blood samples were withdrawn from tail vein under mild anaesthesia at the intervals of 0.5, 1, 3, 6, 9, 12, 18, 24, 36 and 48 h respectively. The withdrawn blood samples were transferred to tubes containing EDTA. Furthermore, samples were subjected to protein precipitation and analyzed using validated HPLC method.

In vitro cytotoxicity studies

The cytotoxic activity of MX and MX loaded nanoparticles were evaluated at various concentrations in HT29 cell lines. In Brief, HT29 cells were cultured at a density of 1×10^3 cells per well in flat-bottomed 96-well plates. After 72 h of treatment with formulations at 0.001, 0.01, 0.1, 1, 5, 10 and 20 µM, MTS solution was added and incubated for 2 h and the cell viability was determined at absorbance at 490 nm using an ELISA reader.

RESULTS

HPLC analysis of MX

MX was analyzed using Shimadzu HPLC C-18 (250 mm x 4.6 mm, 5µm), mobile phase acetonitrile: water (65:35 v/v), and the data was shown in Table 1.

The calibration curve was prepared to calculate the drug concentration. The relative standard deviation is less than 2% indicates the precision of the HPLC analyses. The correlation coefficient was found to be 0.999 and found to be linear relationship between concentration and area. The chromatograms were shown in the Figure 1.

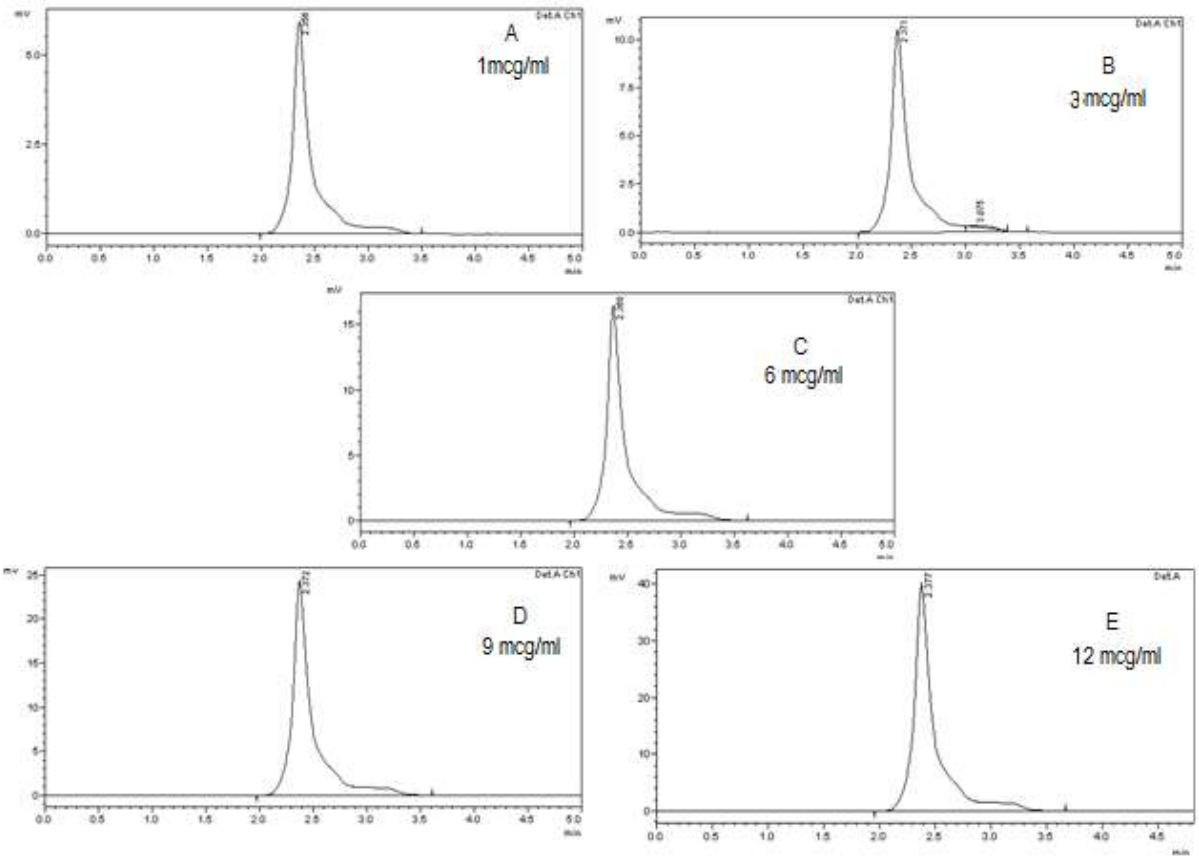

Fig-1: The HPLC chromatograms of meloxicam A (1µg/mL), B (3µg/mL), C (6µg/mL), D (9µg/mL), E (12µg/mL) analyzed with acetonitrile:water (65:35 v/v)

Table-1: Calibration data of the MEL in selected solvent system (*N* = 9) †

Drug concentration (µg/mL)	Area (± S.D.[a])	% R.S.D.[b]
1	76721.8 ± 1287.7	1.73
3	225405. 9 ± 3234.9	1.78
6	427272.8 ± 1278.7	1.14
9	640415.7 ± 9563.9	0.38
12	825479.4 ± 16544.8	1.62

† Two standard stock solutions.
a Standard deviation.
b Relative standard deviation or Coefficient of variance

Preparation and Characterization of the nanoparticles

Two formulations of MX nanoparticles were prepared and the dynamic light scattering technique was adopted for the characterization of the nanoparticles formulation F1 and F2. The mean particle hydrodynamic diameter of F1 and F2 was 174±12 nm and 220±17 nm respectively. The particle size distribution of MX nanoparticles was shown in the Figure 2.

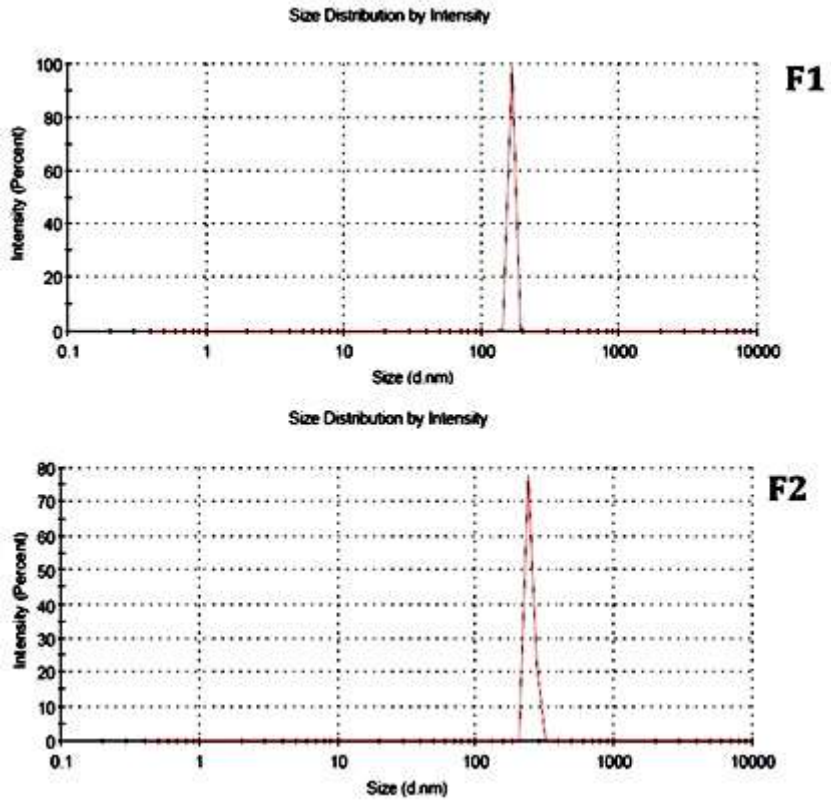

Fig-2: Particle size distribution of meloxicam loaded chitosan four nanoparticle formulations. 3% chitosan (F1), 5% chitosan (F2) with 2% Meloxicam in each formulation

Determination of MX loading capacity and efficiency

As the concentration of MX increases, the size of the nanoparticles was increased. Differences of loading capacity (LC) and loading efficiency (LE) with the different polymer concentrations were shown in the figure 2. The formulation with the higher MX concentration of 0.8 mg/mL has shown maximum loading capacity (65%). The details were shown in the Figure 3.

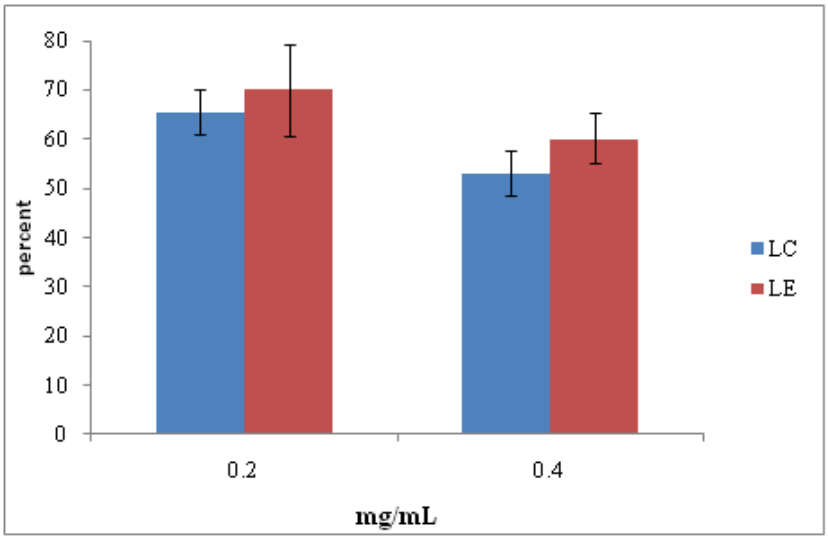

Fig-3: Loading Capacity and loading efficiency study of MX loaded chitosan nanoparticles % LC=percentage loading capacity. %LE= Loading efficiency. F1 and F2 MX Loaded chitosan nanoparticle formulations (n=4, single ANOVA, p<0.05*)

Stability study of MX and nanoparticles at different pH

MX and nanoparticles of MX i.e. F1 and F2 were subjected to pH 3.5, 5.5, 6.8 and 7.4. at diffeernet time internals. At P^H-3.5 (2h, 4h 6h and 24h), the % of drug remaining in F1 were 99.95, 79.35, 78.95 and 41.04. At P^H-5.5 (2h, 4h 6h and 24h), the % of drug remaining in F1 were 99.98, 99.03, 90.37 and 90.19. At P^H-6.5 (2h, 4h 6h and 24h), the % of drug remaining in F1 were 99.98, 98.05, 92.83 and 93.79. Similarly at P^H-3.5 (2h, 4h 6h and 24h), the % of drug remaining in F2 were 98.73, 87.11, 57.19 and 41.05. At P^H-5.5 (2h, 4h 6h and 24h), the % of drug remaining in F2 were 98.72, 94.71, 81.73 and 77.86. At P^H-6.5 (2h, 4h 6h and 24h), the % of drug remaining in F2 were 98.67, 94.04, 96.50 and 99.99. The details were graphically shown in the Figure 4.

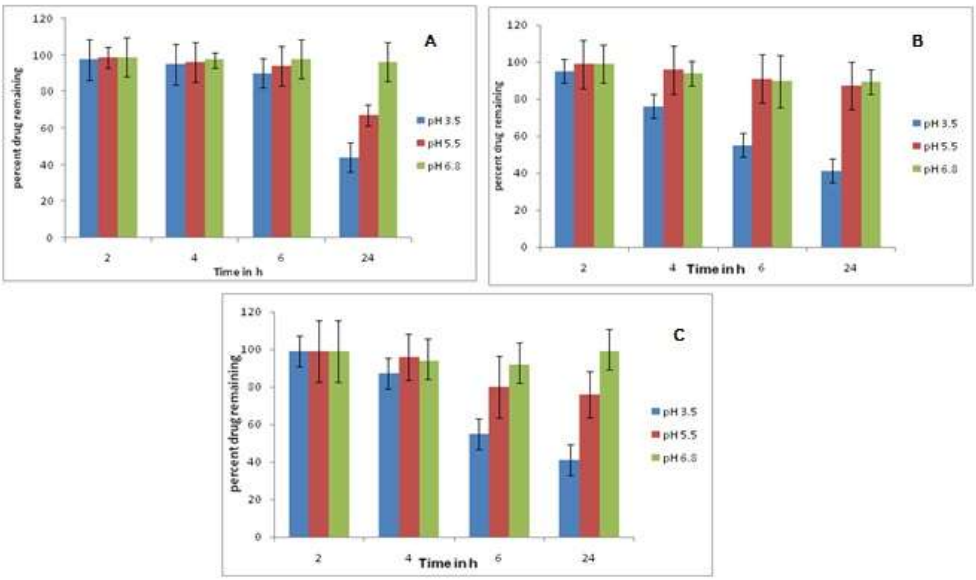

Fig-4: Stability study results of meloxicam loaded chitosan nanoparticle formulations at different P^H A) 3% chitosan. B) 5% chitosan with 2% Meloxicam in each formulation. (n=4, single ANOVA, p<0.05*)

In vitro release of MX from nanoparticles

In vitro release of drug from polymer matrix (F1 and F2) was evaluated for the batch to batch uniformity of drug product and to observe any change in process parameters. The F1 shows the % of drug release at 0, 10, 20, 30, 40, 50, 80, 120, 150 and 200 min were 0, 0.32, 3.20, 7.50, 19.46, 29.55, 38.68, 46.45, 49.65 and 62.56 respectively. Similarly the F2 shows the % of drug release were 0, 0.18, 3.15, 19.06, 21.13, 21.73, 25.36, 39.76, 45.64 and 49.55 respectively and graphically shown in Figure 5.

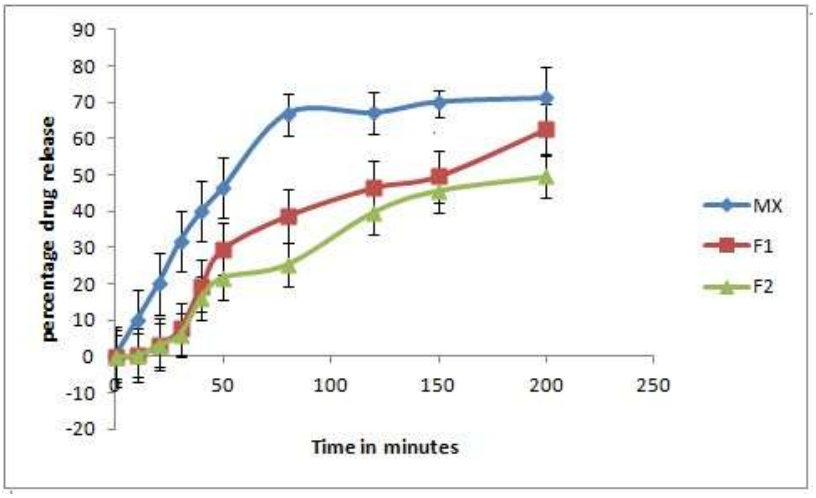

Fig-5: The in vitro drug dissolution profile of MX and MX loaded chitosan nanoparticles. (n=4, single ANOVA, p<0.05*)

In vivo Pharmacokinetic study

This study was conducted using wister rats through oral absorption of both the formulations. The drug content of F1 were found in the plasma samples were 0, 2020, 4472, 7693, 6360,5862,5366 5261, 5075 and 4980 ng/ml at 0, 3, 5, 8,10,12, 18, 25, 35 and 45 min respectively. Similarly the drug content of F2 were found in the plasma samples were 0, 2116, 4620, 8765, 6423, 5762, 5267 5013, 4982 and 4864 ng/ml at 0, 3, 5, 8,10,12, 18, 25, 35 and 45 min respectively. The calculated parameters were shown in Figure 6 and Table 2.

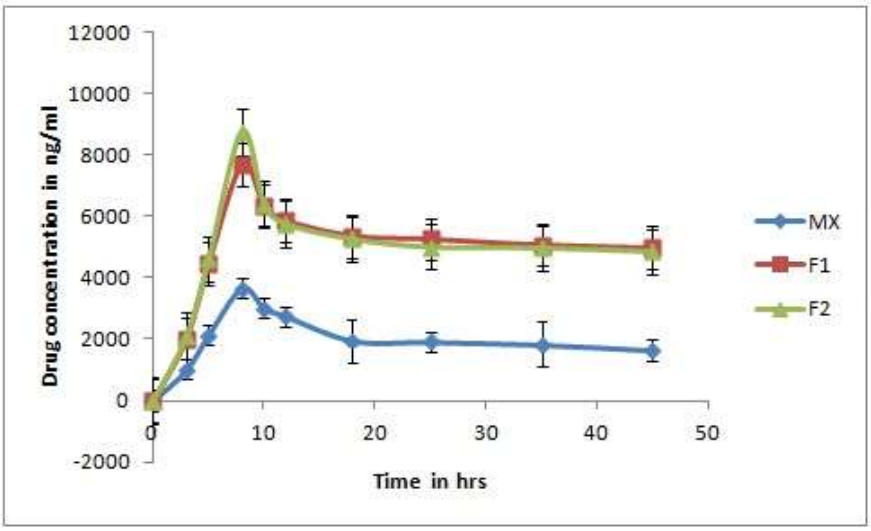

Fig-6: The oral absorption profile of MX, and MX loaded chitosan formulations (F1 and F2) in wistar rats (n=4, single ANOVA, p<0.05*)

Table-2: Pharmacokinetic parameters of MX, F1 and F2 after oral administration

Pharmacokinetic parameter	MX	F1	F2
AUC_{0-t} (µg h/mL)	67.9	240.7	229.8
$t_{1/2}$ (h)	10.36	15.29	12.12
C_{max} (µg/mL)	2.5	7.5	6.7

In vitro cytotoxicity studies

HT29 cell lines were used to estimate in vitro anticancer activity of MX, F1 and F2 formulations using SRB assay. After 24 hours incubation in HT29 cell lines with MX, F1 and F2 formulations. Furthermore, supernatant was taken out and washed with PBS and images were captured at 40X. The MX and formulation treated cells are given in Figure 7. The growth curve was shown in the Figure 8.

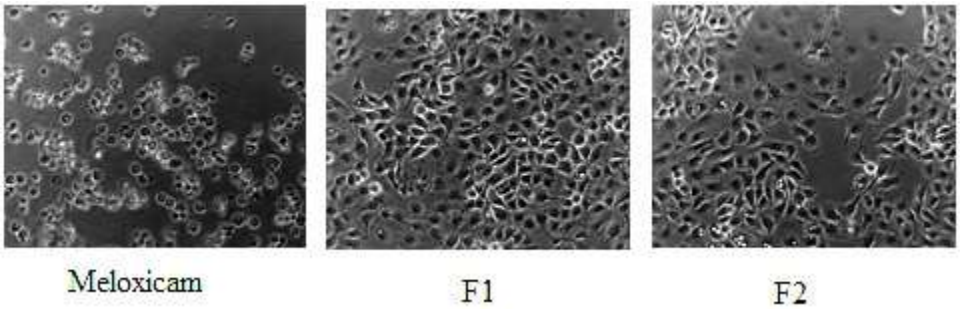

Fig-7: The HT29 colon cancer cell lines treated with MX and MX loaded chitosan formulations (F1and F2)

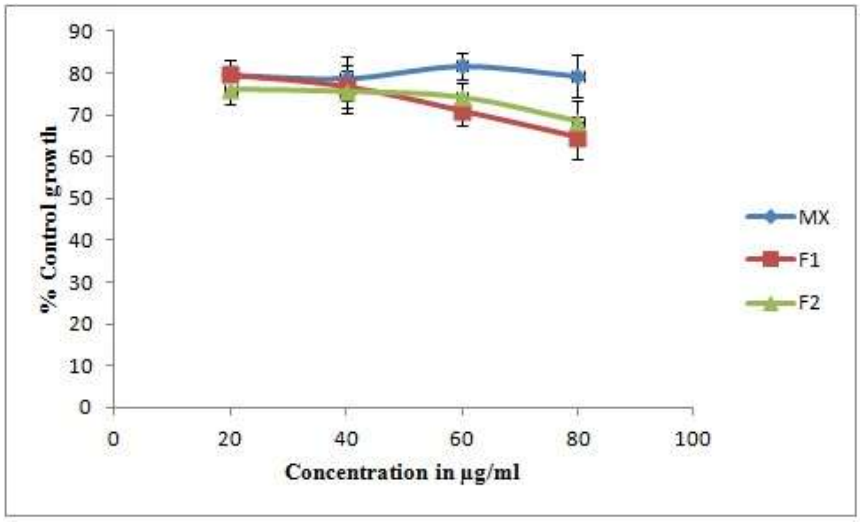

Fig-8: Growth Curve of meloxicam and four chitosan loaded formulations (F1 and F2) on HT29 colon cancer cell lines. (n=4, single ANOVA, p<0.05*)

DISCUSSION

The HPLC analysis of meloxicam was conducted to determine the % of drug release for the prepared solutions of both the formulations, F1 and F2. The % of drug release at different time interval were calculated from the calibration graph of meloxicam. The values were found satisfactory. The characterization of both F1 and F2 using dynamic light scattering technique reveals the size distribution was proper. The loading capacity and efficiency of both formulations indicates that by increasing drug loading, loading efficiency decreased and loading capacity was increased. Stability study of nanoparticles of both formulations at different pH was conducted and reveals that the formulation F2 was showed highest stability compared to F1. The pH stability of the formulation is very important for the drug content maintenance in the stomach and intestine. Furthermore, the stability at pH 6.8 is essential for the drug absorption while drug formulation residence in the GIT. Chitosan was showed good stability at pH 6.8 and maintained for 24 h period. The determination of the release of drug substance from the polymer and plays an important role in quality controls. F1 and F2 were shown different drug release profile. F1 and F2 were shown sustained drug release pattern. The blood samples were analyzed using validated HPLC method. The formulations F1 and F2 were shown higher drug release compared to MX alone. The peak plasma concentrations were reached at 6 h and further the concentration of MX was started decreasing drastically. MX alone did not release effectively throughout the absorption phase compared to both formulations. *In vitro* cytotoxicity study was conducted and found that the cells treated with formulation F1 and F2 have showed faintly induced cell death in HT29 cell lines. F1 was showed better cytotoxcity over F2. The cells were relieved membrane blebbing and granules.

CONCLUSION

The study confirmed that meloxicam loaded chitosan nanoparticle formulations has showed superior bioavailability and pH stability. Among two formulations F1 has shown slightly higher cytotoxicity over other formulations when compared to meloxicam alone, hence meloxicam loaded chitosan loaded nanoparticles can be considered to be a promising system for delivery of meloxicam. These nanoparticle formulations are effective in the treatment of colon cancer compared to other chemotherapeutic agents which will lead to fewer side effects. Further study is required to evaluate the formulation parameters *in vivo* and improved cytotoxicity with other polymers.

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CONFLICTS OF INTEREST

No potential conflict of interest was reported by the authors

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