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FORMULATION, EVALUATION AND CYTOTOXIC POTENTIAL OF METRONIDAZOLE LOADED POLOXAMER 407 HYDROGEL IN SCC-29 CELL LINES

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ABSTRACT: The present research work was planned to formulate poloxamer 407 based hydrogel formulations of metronidazole and the evaluation of various parameters like swelling behavior, drug PH stability, *in-vitro* and *in-vivo* drug release, and *in-vitro* cytotoxic activity. Two different concentrations of metronidazole hydrogel formulations were prepared using poloxamer 407 and were assessed by a validated HPLC method for drug content, pH stability, and *in-vivo* drug release. Further, *in-vitro* anticancer activity was evaluated using sulphorhodamine B (SRB) assay in SCC29 cell lines. Both the formulations F1 and f2 showed better pH stability at pH 3.5, 5.5 and 6.8. The formulation F1 was able to absorb about 152% of its weight of water within 80 min, whereas F2 absorbed 167.4% of its weight of water and remains constant over 100 min. *In-vitro* and *in-vivo* drug release pattern showed half-life at 6 h, AUC_{0-t} 692 and 684ng h/ml, C_{max} 1059 and 1142 ng/ml for F1 and F2 respectively. Hydrogel formulation F1 showed improved percentage control growth when compared to F2 hydrogel formulation and metronidazole alone.

INTRODUCTION: Metronidazole (MT) which is chemically 5 nitroimidazole derivatives with the molecular weight of 171.156, gm/mol and with a molecular formula $C_6H_9N_3O_3$. It is a nitroimidazole which is used for the treatment of vaginitis, amebiasis, giardiasis, trichomonas infections and several anaerobic bacterial infections [1]. It shows antibacterial and protozoal activities, by converting itself active intermediate product in reduced form and which breaks DNA strands, thereby inhibiting DNA synthesis and bacterial cell growth [2].

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Ploxomer 407 have been currently received major attention in the field of thermosensitive hydrogels. It is an amphiphilic synthetic copolymer which consisting of a hydrophobic poly (Oxypropylene) (POP) block between two hydrophilic poly (Oxyethylene) (POE) blocks [3-5]. Because of its amphiphilic nature, these molecules can make self-assemble readily to form micelles base on the temperature and concentration.

These hydrogels have been characterized by their ability to carry a significant amount of drug. They are also, nontoxic biodegradable and stable, therefore suitable for uses in controlled release formulations [6]. Extensive literature review on metronidazole revealed that, along with its antiprotozoal and antibacterial activities, the cytotoxic property of MT was also reported.

The cytotoxic activity of metronidazole (Flagyl) and imidazole (MISO) was conducted on hypoxic Chinese hamster ovary cells, and considerable cytotoxic activity was reported [7]. In another reported article it was evaluated for its cytotoxic activity on DLD-1 colorectal cancer cell lines. They reported that the cell viability was decreased in all experimental groups when compared to control and found statistically significant [8]. MT also proved its cytotoxic potential on breast cancer cell lines, Sadowski *et al*. reported the impact of cytotoxicity of MT on MCF-7 and MDA-MB-231 breast cancer cell lines [9]. Based on the above-reported studies the present research work was programmed to prepare hydrogel formulations of MT using poloxamer 407. An extensive review of literature also revealed that till there were no hydrogel formulations of MT has been tested for its cytotoxic property, therefore in this present study we compared the cytotoxic property of MT and MT and its hydrogel formulations to investigate drug released from the prepared hydrogel show its cytotoxic property.

MATERIALS AND METHODS: Poloxamer-407 (from BASF India Ltd, Mumbai, India) were obtained as a gift sample. Water purified by reverse osmosis, MilliQ, USA and further filtered by 0.22 µm membrane filter. HPLC grade methanol was purchased from SD Fine Chemicals. Pure Metronidazole (99.41% purity) was obtained as a gift sample from Dr. Reddy's Laboratory, Hyderabad, India.

HPLC Analytical Method: The concentration of MT in hydrogel was determined using developed validated analytical method [10]. Accurately weighted 10 mg standard metronidazole was dissolved in 65.0 ml of methanol and made up to 100 ml with Millipore water to obtain the final concentration of 100 µg/mL. From the standard stock solution of metronidazole (100 mcg/ml) different concentrations were prepared in the range of 0.003, 0.00625, 0.0125, 0.025, 0.05, 0.1, 0.3, 0.5, 1.0, 5.0, 10 µg/mL and injected in to HPLC system. Shimadzu LC-20AD HPLC equipped with SPD-20A UV/VIS detector, a Rheodyne (20 µl volume capacity) injector, and a Shimadzu LC Solution software was used. Chromatographic separation was performed on 25 cm RP-C18 (250 mm × 4.6 mm i.d.) with a particle size of 5 µm HPLC column. The mobile phase consisted of methanol, and millipore water (70:30, v/v) was used. The freshly prepared mobile phase was filtered through 0.22 µm membrane filter and degassed for 10 min before analysis. All samples were analyzed at a flow rate of 1.0 ml/min, and effluent was monitored at 365 nm. A 25 µl of sample was injected onto the Rheodyne and analyzed.

Preparation of Metronidazole loaded Poloxomer 407 Hydrogel: Metronidazole loaded hydrogel formulations (F1 and F2) were formulated [11] by dissolving 150 mg poloxamer 407 in Millipore water. Total content was dissolved and freeze it. 10 mg of accurately weighted MT was added to dissolve in the prepared gel base. The required amount of Millipore water was added, kept the total content in the refrigerator until the clear gel was formed to obtained 15% hydrogel formulation (F1). Similarly, 20% hydrogel formulation (F2) was prepared by dissolving 200 mg poloxamer 407 and 10 mg of metronidazole, in the required amount of Millipore water.

Study of Swelling Behavior of Hydrogels in Pseudo Extra Cellular Fluid (PECF): Prepared hydrogels (F1 and F2) were investigated of their swelling properties [12] in PECF solution. The PECF solution and simulated wound fluid which is consists of 0.22 g of KCl, 0.68 g of NaCl, 2.5 g of NaHCO3, and 0.35 g of NaH_2PO_4 in 100 ml of deionized water. The pH of PECF and ionic strength of the solution were 7.4 ± 0.2 and 0.48M respectively. The freshly prepared hydrogel formulations were left to swell in PECF solution at 25 °C. Hydrogels were weighed accurately and then soaked in PECF solution. 10 min later hydrogels were removed from solvent carefully, wiped by filter papers, reweighed, and placed in PECF solution. Until a constant weight was reached for each sample, this procedure was repeated several times. The percentage of swelling of hydrogels was calculated from the difference between the initial and the final weight of the sample divided by the initial weight of the sample.

Stability Study of MT and Hydrogel at Different pH: By using various pH (3.5, 5.5 and 6.8), stability study was conducted for the prepared hydrogels. 0.1 M hydrochloric acid, phosphate buffers were selected. 10 mg of MT was accurately

weighted and MT loaded hydrogels were transferred to 2 ml centrifuge tube and 1 ml of each buffer was added to tubes containing hydrogels and incubated at 25° C for 24 h. HPLC method was adopted to quantify the drug content and the extent of drug degradation was evaluated.

***In-vitro* Release of MT from Hydrogels:** Aliquots of 1 ml of MT loaded hydrogel formulations were centrifuged at 10000 × 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. A tube was selected and centrifuged at 10000 x g for 15 min at a various time interval. The released MT was determined using HPLC analytical method.

Determination of Bioavailability of MT loaded Hydrogel Formulations: To elicit any pharmacological activity the maximum amount of drug should reach systemic circulation. Due to that, MT alone and hydrogels have been administered orally to the wistar rats for the determination of the amount of MT reaching systemic circulation. Balaji Institute of Pharmaceutical Sciences, Narsampet, Warangal, has approved animal facility with CPCSEA registration no.1694/PO/Re/S/14/cpcsea and protocol was approved by the Institutional Animal Ethics Committee (IAEC). 5 mg of MT equivalent to MT and MT loaded hydrogels were taken and administered to male wistar rats (~280 g) orally, and blood samples were withdrawn from the tail vein under mild anesthesia at the intervals of 0.5, 1, 3, 6, 9, 12, 18 and 24 h respectively. EDTA tubes were used to transfer the blood samples, and the samples were subjected to protein precipitation, and quantity was estimated using the validated HPLC method.

***In-vitro* Cytotoxicity Study:** To investigate the cytotoxic activity the SCC 29 cancer cell lines were used which are collected from NCI (National Cancer Institute, USA). These cells were cultured in complete growth medium (RPMI 1640) supplemented with 10% fetal bovine serum (Sigma, USA), 1% 100 U/ml streptomycin (Sigma, USA) and 1% 100 U/ml penicillin at 5% CO_2, 37 ºC, and 97% relative humidity. 75 cm^2 canted-neck tissue culture flask was used to allowed to grow the human cancer cell lines routinely and passage regularly by using trypsin/EDTA.

Further, subculture was performed when a confluence of 90% was reached. Sulforhodamine B (SRB) assay method was utilized for the *in-vitro* cytotoxicity of metronidazole and prepared hydrogel formulations (F1and F2) [13]. As per the standard protocol [24]. Briefly, 5 × 10^3 cells/ well of SCC 29 cells were seeded in 96 well plates and incubated for next 24 h. Various concentrations (10-80 µg/mL) of metronidazole hydrogel formulations were prepared, and the plates were incubated for 48 and 72 h and fixed with cold trichloroacetic acid for 1 h at 4 ºC. The plates were washed 3 times with distilled water and air dried. The SRB dye (0.4%) was added in the plates and kept at room temperature for 30 min. 1% (v/v) glacial acetic acid was used to wash the plates for the removal of unbound SRB dye. The tris buffer (10 mM, pH 10.4) was added to each of the well and allowed to solubilize by keeping on a shaker. A microplate reader (Biotek Synergy HT) at 540 nm was used to measure the values.

RESULTS AND DISCUSSION:

HPLC Analysis of Metronidazole: Metronidazole was analyzed using Shimadzu LC-20AD HPLC equipped with SPD-20A UV/VIS detector, a Rheodyne (20 µl volume capacity) injector, and a Shimadzu LC Solution software was used., mobile phase Methanol : water (70:30 v/v), and the data was shown in **Table 1**. The calibration curves were prepared to determine the drug concentration. The relative standard deviation is less than 2% indicates the precision of the HPLC method. The correlation coefficient was found 0.999 and indicated the linear relationship between concentration and area. The chromatogram at 15 µg/Ml was shown in **Fig. 1**.

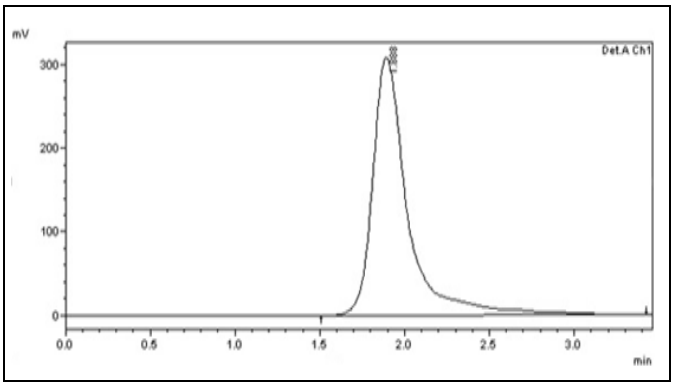


FIG. 1: CHROMATOGRAM OF METRONIDAZOLE AT 15 µg/mL USING MOBILE PHASE METHANOL: WATER (70:30 v/v)

TABLE 1: CALIBRATION DATA OF THE METRONIDAZOLE IN SELECTED SOLVENT SYSTEM (N=9)[b]

Concentration (µg/mL)	Peak area (mean)	% RSD[a]
0.1	68005	1.21
0.5	344493	0.73
1	691833	0.36
3	2065511	0.64
5	3366894	0.59
8	5600710	0.72
12	8351507	0.62
15	10283225	0.33

[a]Relative SD or coefficient of variance. [b]Two standard stock solutions

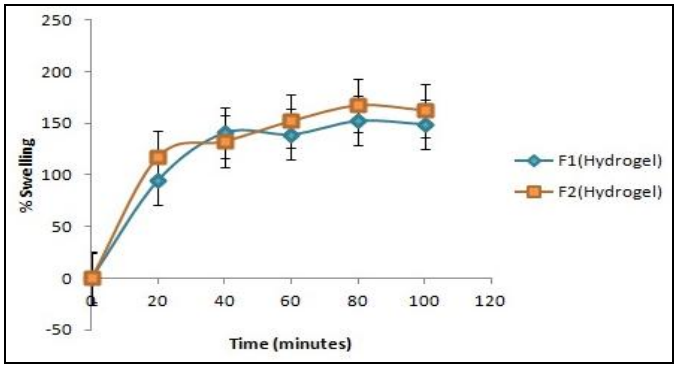


FIG. 2: SWELLING BEHAVIOUR OF MT LOADED HYDROGEL FORMULATIONS (F1 AND F2)
(n=4, single analysis of variance, P<0.05*)

Swelling Behaviour of Hydrogels in PECF: The percentage swelling of metronidazole hydrogel formulations were calculated after pre-determination duration in PECF solution which was plotted as a function of time shown in **Fig. 2**. Swelling behavior of hydrogel formulations, F1 and F2 were checked in PECF solution as described earlier. The formulations were able to absorb about 152% of its weight of water (F1) within 80 min, whereas 167.4% of its weight of water (F2) and remains constant over 100 min for both the formulations. After that, the swollen film was broken into pieces.

Stability Study of MT and Hydrogels at Different pH: Metronidazole and its hydrogel formulations (F1 and F2) were treated with pH 3.5, 5.5 and 6.8 at different time internals (2h, 4h, 6h, and 24 h). At pH-3.5 (24 h), the % of drug remaining in F1 was 43.17. At pH-5.5 (24 h), the % of drug remaining in F1 was 85.44. At pH-6.5 (24 h), the % of drug remaining in F1 was 86.03. Similarly, At pH-3.5 (24 h), the % of drug remaining in F2 was 42.19. At pH-5.5 (24 h), the % of drug remaining in F2 was 77.01. At pH-6.8 (24 h), the % of drug remaining in F2 was 83.75. The details were graphically shown in **Fig. 3**. The pH stability of the formulation is prime for the drug content maintenance in the stomach and intestine. Further, the stability at pH 6.8 is essential for the drug absorption and drug formulation residence in the GIT.

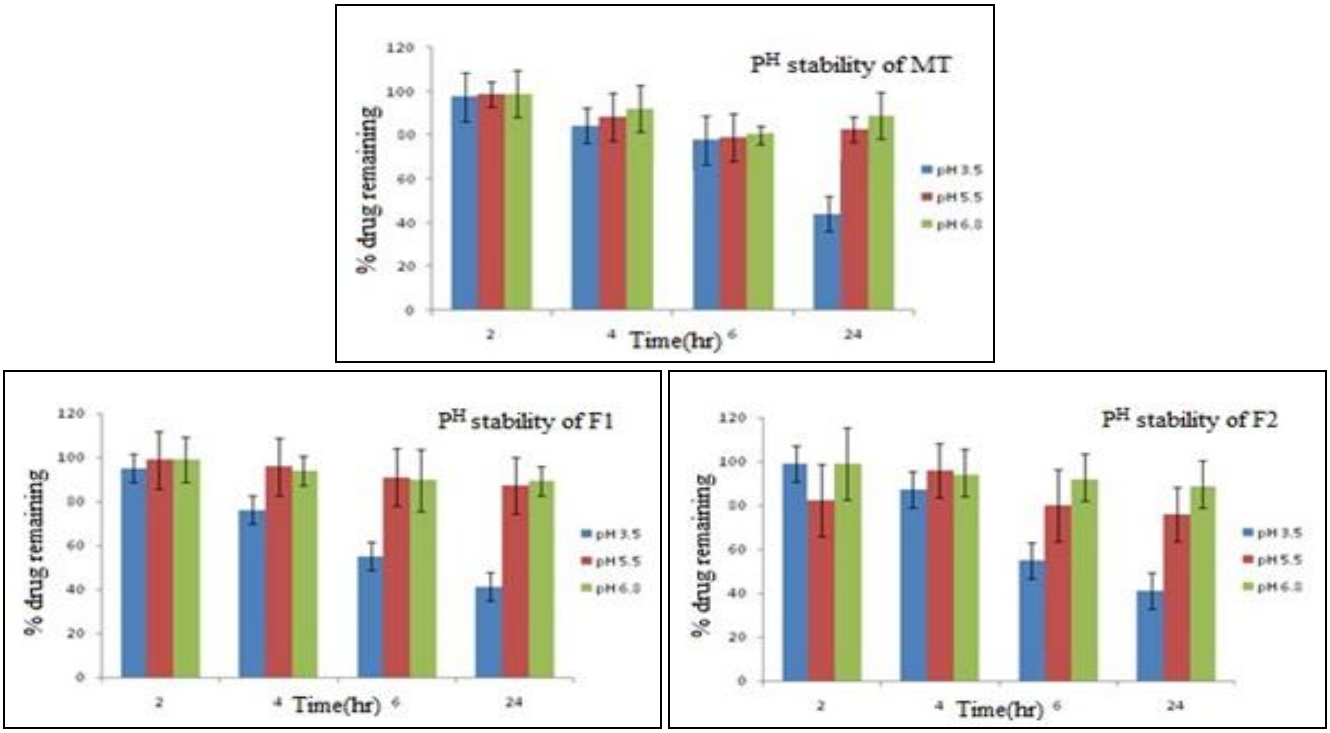


FIG. 3: STABILITY STUDY OF METRONIDAZOLE HYDROGELS FORMULATIONS AT DIFFERENT pH
(n=4, single ANOVA, p<0.05*)

***In-vitro* Release of MT from Hydrogel Formulations:** *In-vitro* releases of the drug from the hydrogel formulations (F1 and F2) of MT, were evaluated for the batch to batch uniformity of drug product and to observe any change in process parameters. % of drug release for F1 at 0.5, 1, 2, 4, 6 h were 35.23, 43.46, 61.3, 64.92 and 69.6 respectively. Similarly, the % of drug release for F2 was 31.12, 32.3, 42.6, 46.32 and 51.11 respectively. The drug release pattern was shown in **Fig. 4**.

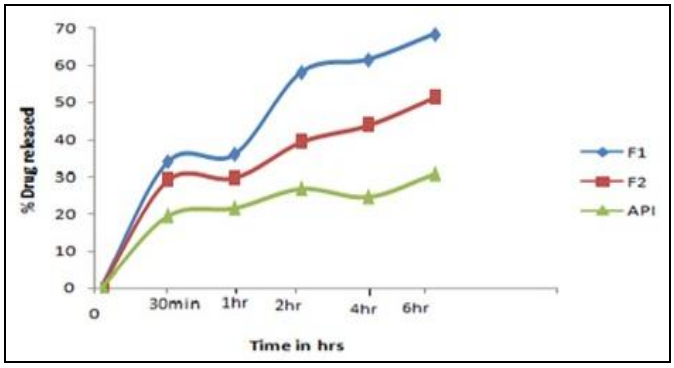


FIG. 4: THE *IN-VITRO* DRUG DISSOLUTION PROFILE OF MT AND MT LOADED HYDROGEL FORMULATIONS

***In-vivo* Pharmacokinetic Study:** It was conducted using wistar rats through oral administration of both the formulations. The drug content of F1 was found in the plasma samples were 280.4, 560.7,

955.3, 730.4, 320.5, 205.5, and 122.3 ng/mL at 0.5, 1, 3, 6, 9, 12, 18 and 24 h respectively. Similarly, the drug content of F2 was found in the plasma samples were 332.4, 437.3, 527.6, 1007.1, 643.6, 407.8 and 174.3 ng/mL at 0.5, 1, 3, 6, 9, 12, 18 and 24 h respectively. The calculated parameters were given in **Fig. 5** and **Table 2**. Validated HPLC method which was described earlier was utilized to analyze the blood samples. Both the formulations were shown higher drug release compared to MT alone. The peak plasma concentrations were observed at 6h, and further, the concentration of MT was started in decreased order. MT alone did not release effectively throughout the absorption phase when compared to both formulations F1 and F2

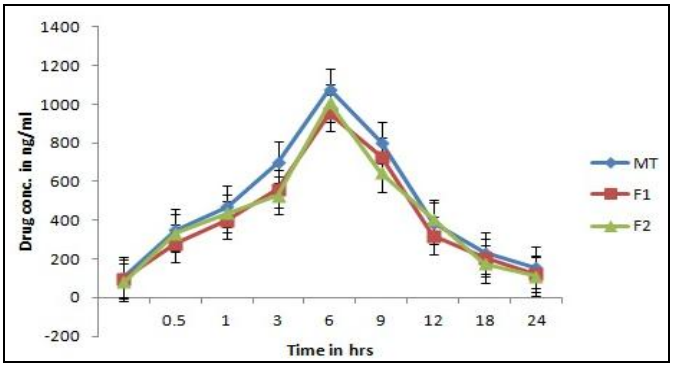
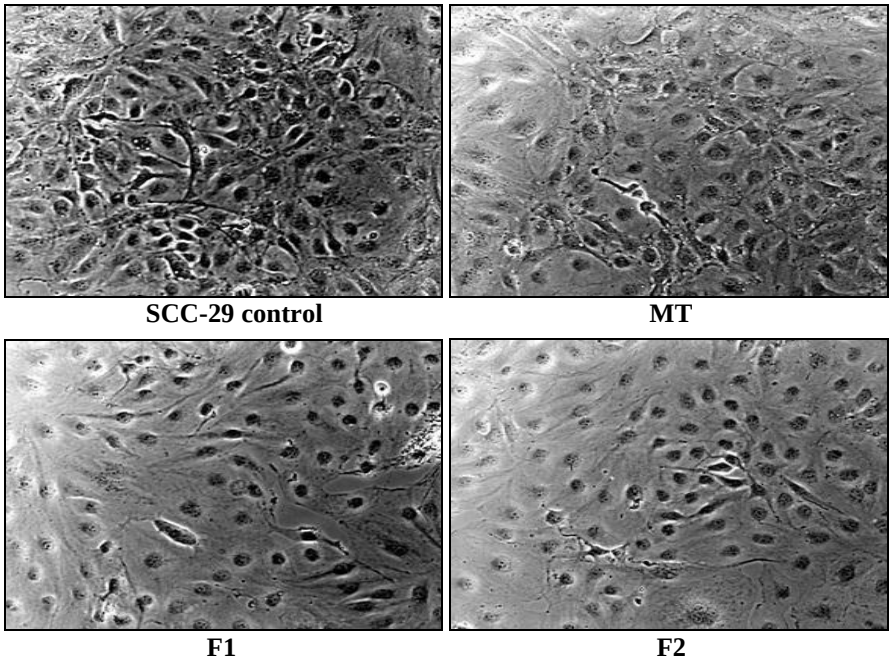


FIG. 5: THE PHARMACOKINETIC PROFILE OF MT AND MT LOADED HYDROGEL FORMULATIONS (F1AND F2) IN WISTAR RATS (n=4, single ANOVA, $p<0.05$*)

TABLE 2: PHARMACOKINETIC PARAMETERS OF MT, F1, AND F2 AFTER ORAL ADMINISTRATION

Pharmacokinetic parameters	Metronidazole	F1	F2
AUC_{0-t} (ng h/ml)	785	692	684
$t_{1/2}$ (h)	6	6	6
C_{max} (ng/ml)	1432	1059	1142



SCC-29 control **MT**

F1 **F2**

FIG. 6: THE SCC-29 COLON CANCER CELL LINES TREATED WITH MT AND HYDROGEL FORMULATIONS (F1-F2)

***In-vitro* Cytotoxicity Study:** The cytotoxicity of MT and both the hydrogel formulations F1 and F2 were evaluated to investigate the effects on SCC-29 cells. Cell viability was determined using the SRB assay. The supernatant component was taken out and washed with PBS and images were taken under 40X. The MT and hydrogel formulations treated cells are showed in **Fig. 6**. The cells applied with formulations F1 and F2 showed better anticancer activity over MT alone. Whereas, the formulation F1 have shown faintly induced cell death in SCC-29 cell lines. F1 was showed quite better cytotoxicity over other F2. The cells were showed blebbing and granules. The growth curve was shown in **Fig. 7**.

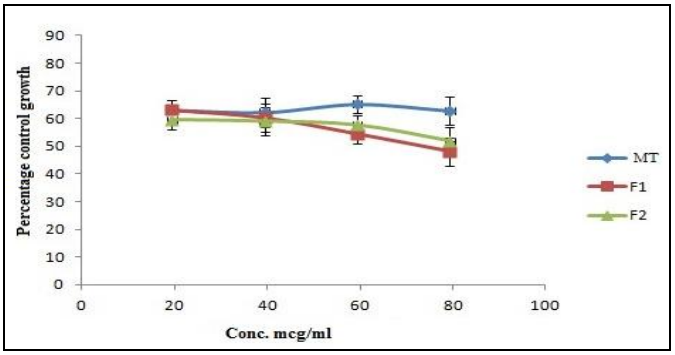


FIG. 7: GROWTH CURVE OF METRONIDAZOLE AND TWO HYDROGEL FORMULATIONS (F1-F2) ON SCC 29 CELL LINES. (n=4, single ANOVA, $p<0.05$*)

CONCLUSION: Rely on empirical evidence the present study confirmed that prepared metronidazole hydrogel formulations had shown better bioavailability and pH stability. Among two formulations F1 has shown little better cytotoxicity over F2 formulation when compared to metronidazole alone. Hence, metronidazole loaded hydrogel formulations can be considered to be a promising system for the delivery of metronidazole for cytotoxic potential.

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CONFLICT OF INTEREST: The authors have no conflict of interest.

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