



## Research Article

# Guar Gum Micro-particles for Targeted Co-delivery of Doxorubicin and Metformin HCL for Improved Specificity and Efficacy Against Colon Cancer: *In Vitro* and *In Vivo* Studies

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**Abstract.** Doxorubicin and Metformin HCL is a known chemotherapeutic combination that wipes out tumors and prevents their recurrence. However, limited site specificity confines its application. Here we report Doxorubicin and Metformin HCL-loaded guar gum micro-particles prepared by emulsification cum-solidification method. Developed micro-particles were characterized as spherical shape particles with smooth surface and micro size diameter. Encapsulation of drugs in combination was confirmed by their characteristic functional groups (FT-IR), change in phase transition temperature (DSC) and X-ray diffraction pattern (XRD). Particles were observed to be stable at 25 and 5°C. The *in vitro* Doxorubicin and Metformin HCL release study in simulated gastric (SGF), intestinal (SIF) and colonic fluid (SCF) confirms restricted release in SGF (9.3 and 9.6%, respectively, in 2 h) and SIF (10.8 and 14.7%, respectively, in the next 3 h) and highest release in SCF (about 68 and 73.3%, respectively) in colon. Developed micro-particles showed 78% recovery in tumor volume and considerable improvement in histological changes. X-ray images confirmed good target ability of micro-particles to colon. In conclusion, the specially designed, stable micro-particles are able to target drug combination to colon and improve efficacy by ensuring maximum drug release in colon as compared with Doxorubicin and Metformin HCL combination.

**KEY WORDS:** micro-particles; colon targeting; guar gum; Doxorubicin; Metformin HCL; colon cancer.

## INTRODUCTION

Colorectal cancer (CRC) is the most usual cancer in women and men both. In 2018, 6.1% (1,096,601) cases were of CRC only out of all 18.1 million new cancer cases. About 551,269 (5.8%) deaths were recorded of all cited deaths worldwide due to all types of cancers. It has become the fourth dominant cause of cancer-related mortalities, with around 6% cancer related deaths per year (1). Colon cancer (CC) develops as the result of the changes in the standard colon epithelium in an adenomatous polyp. It has been described that genetic modification plays vital role in cancer development, which has shown gradual growth in recent decades (2).

Doxorubicin is a byproduct of actinobacteria *Streptomyces peucetius* var. *Casieus* (3) and is a drug of anthracycline class. It has widely been investigated as chemotherapeutic agent against

CC and is repeatedly used against other types of cancers also including soft tissue sarcomas and hematological malignancies (4). Despite immense utility as chemotherapeutic agent, doxorubicin is cardiotoxic and cancerous cells show resistance against it (5). Therefore, these limitations are the major obstacle in its comprehensive applications (6). However, it is still considered as good option in chemotherapy of CC.

Reports suggest anti-cancer property of Metformin HCL in addition to its anti-diabetic effect. It acts specifically against primary cancers like lung, pancreatic, colon, ovarian, breast and many more. However, Metformin HCL is less known for its effect in recurrent cancer like CRC that affects nearly half of the patients treated by conventional chemotherapies (7). Metformin HCL has been shown to sensitize multidrug resistant cancers like breast (8) and colon cancer (7). It has been reported to cure cancer and prevent cancer recurrence (associated with Doxorubicin therapy) when combined with doxorubicin however, sole Metformin HCL has no effect on the same (9). In addition, it exerts cardioprotective actions and counter cardiotoxic effects of Doxorubicin (5). Several reports cite the higher demand of glucose in cancerous cells for normal growth (10) where Metformin HCL may help reduce the glucose level also so that the survival of the cancerous cell can be restricted.

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However direct oral administration of this combination may lead to cytotoxicity and organ toxicity; therefore, site specific delivery directly to colon could be the best way to avoid the situation (11). In doing so, various types of drug delivery systems have been developed with immense potential to release drugs specifically to the colonic region. Out of biodegradable polymers, pH-sensitive polymers (12), pro-drugs, timed deliver systems, biodegradable inserts and hydrogels (13), the acceptance of polysaccharides (pectin, guar gum, chitosan and amylase)-based targeted carries are very high (14) because polysaccharides help bypass physiological environment of stomach and small intestine, and, once been available to the colon, they start reacting to the bacterial environment of colon (15). Bioadhesive and biodegradable nature of guar gum make it polymer of choice for developing controlled and colon targeted drug delivery systems (16).

Guar gum obtained from the seeds of *Cyamopsis tetragonolobus* consists of linear chains of (1→4)-β-D-mannopyranosyl units with α-D-galactopyranosyl units attached by (1→6) linkages (17). Its gelling property retards the release of the drug from the dosage form and makes it susceptible to degradation in the colonic environment (18). Therefore, we hypothesized that developing guar gum micro-particles for targeted delivery of Doxorubicin and Metformin HCL to the colon would be the most suitable option as they could lead to the enzymatic activities in microbial flora (act as a release trigger) rich colonic region. Furthermore, this technology was supposed to solve the purpose best in efficient chemotherapy of CRC.

## MATERIALS AND METHODS

### Material

Doxorubicin was kindly obtained as a gift sample from Fresenius Kabi Oncology Ltd, Baddi, India. Metformin HCL was kindly provided as a gift sample by Psycho Remedies Limited, Ludhiana, Punjab. Guar gum, tween 80 and glutaraldehyde were purchased from Central Drug House Pvt. Ltd., Mumbai, India. Light liquid paraffin was purchased from Nice Chemicals Pvt. Ltd. Span 80, barium sulphate and hydrochloric acid was purchased from Loba Chemie Pvt. Ltd. All the chemicals used in this investigation were of analytical grade and were used as received.

### Ethics Statement

Wistar rats (either sex) of  $252 \pm 5$  g average weight were procured from the animal house of ISF College of pharmacy, Moga, Punjab, India. Procured animals were housed under standard laboratory condition ( $25 \pm 3^\circ\text{C}$  room temperature and 60% humidity along with 12 h light and 12 h dark cycle) with free access to food and water *ad libitum*. All the animal experiments were carried out as per the ARRIVE guidelines, the EU Directive 2010/63/EU for animal experiments. This study was duly approved by the CPCSEA, through the Institutional Animal Ethics Committee (Protocol no: ISFCP/IAEC/CPCSEA/24/2019/407).

## Method of Preparation of Guar Gum Micro-particles

Micro-particles were prepared using emulsification cum-solidification method as the procedure reported elsewhere (19) with some modifications. Brief, light liquid paraffin was used as a dispersion medium and glutaraldehyde as a cross-linker. About 4% w/v guar gum (polymer) solution was prepared in 0.2% w/v aqueous solution of tween 80. Doxorubicin (0.03% w/v; Drug 1), Metformin HCL (0.06% w/v; Drug 2) and Metformin HCL (0.06% w/v) and Doxorubicin (0.03% w/v) combinations were used to prepare three deferent types of micro-particles. Drugs were mixed in the guar gum solution by ultra sonication using probe sonicator, and the mixture was allowed to swell for 2 h. Further the drug and polymer solution was added drop by drop with the help of syringe into 150 mL of liquid light paraffin (organic phase) containing 0.5% w/w span 80 (emulsifier) maintained at  $60^\circ\text{C}$ . This dispersion was stirred using a mechanical agitator at 2000 rpm for 20 min. After complete dispersion of polymer-drug solution into continuous oil phase, concentrated hydrochloride acid (0.2 mL) was added to the dispersion. Simultaneously, 0.2 mL of glutaraldehyde was added into dispersion and stirring was continued for 2 h at constant speed of 2000 rpm. After the completion of the cross linking reaction, micro-spheres were collected by centrifugation. These were washed 3 times with n-hexane, methanol and acetone to remove traces of oil. For the removal of drugs and cross linking agent, micro-particles were washed with buffer solution of pH 1.2. Finally, micro-particles pellet was allowed to dry at room temperature and dried in a vacuum desiccator for 48 h. The particles were left as such in desiccators until further use (20).

## Characterization of Micro-particles

### Determination of Particle Size

Particle size of the developed micro-particles was measured using Zeta sizer (Delsa Nano particle Analyzer). In doing so, micro-particles dispersion was taken in the cuvette and the particle size was observed using the software provided by the manufacturer (21).

### Shape and Surface Morphology

The morphology and appearance of the developed micro-particles were examined by scanning electron microscopy (SEM). This analysis was done by sprinkling powdered micro-particle sample on double adhesive tape, which stuck to an aluminum stub. The stubs were then coated with gold to a thickness of  $\sim 300$  Å using a sputter coater, and the photographs of samples were taken by SEM at  $1000\times$  (21).

### Drug Loading and Entrapment Efficiency

Drug loading and entrapment efficiency were investigated as per the protocol reported elsewhere using back calculation method (22). Micro-particle dispersion was centrifuged at 12000 rpm for 30 min. Supernatant was collected in a test tube separately. Pellet was further washed 3 times using water, centrifuged and supernatants were collected. Drug loading and entrapment efficiency were determined by

analyzing total peak area of the drugs present in all the supernatants using HPLC (Alliance, Waters). These parameters were calculated using the following formula:

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

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

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

#### Drug–Polymer Interaction

**Modification in Functional Groups of Doxorubicin and Metformin HCL.** Fourier transform infrared spectroscopy (FTIR) of dried powdered sample Doxorubicin, Metformin HCL and micro-particles was done by making potassium bromide (KBr) disk of drugs and micro-particles separately. Mixture of 2 mg samples and 98 mg KBr was thoroughly triturated by pestle and mortar and compressed in a vacuum press to obtain a disk. Infrared spectrum was recorded by scanning over a wave number region of 500–4000 cm using Nicolet omnic software. The characteristics IR spectrum and peaks were observed and were compared with the reference spectrum of Doxorubicin and Metformin HCL (23).

**Phase Transition Temperature of Doxorubicin and Metformin HCL in Micro-particles.** Differential Scanning Calorimetric (DSC) was used to study the degree of polymorphic phase transitions temperature of Doxorubicin and Metformin HCL in final formulation (Fig. 1). DSC (NETZSCH, DSC 200 F3, Maia) of Doxorubicin, Metformin HCL and micro-particles of Doxorubicin and Metformin HCL was performed in perforated aluminum-sealed pans at a heating rate of 5°C/min from 30 to 400°C using nitrogen as blanket gas (50 mL/s) (24).

**Physical State of Doxorubicin, Metformin HCL and Their Micro-particles.** X-ray diffractometer (XRD) was used to study the physical state of Doxorubicin, Metformin HCL and their micro-particles in terms of crystallinities (Fig. 2). XRD analyzed the diffraction patterns of the powdered samples when these were exposed to a monochromatic nickel-filtered copper radiation (45 kV, 40 mA) in a wide-angle X-ray diffractometer (D8 Advance, BRUKER, Germany) with scanned angle (24,25).

### Evaluation of Micro-particles

#### *In Vitro* Drug Release from the Developed Micro-particles

*In vitro* release study of Doxorubicin and Metformin HCL from developed micro-spheres (Doxorubicin and Metformin HCL micro-particles) was carried out using

USP dissolution apparatus (Lab India Pvt. Ltd, Navi Mumbai) type I at 50 rpm in 900 mL of simulated fluids at  $37 \pm 0.5^\circ\text{C}$ . Dissolution profile of Doxorubicin and Metformin HCL micro-particles were compared with their respective solutions. Aqueous dispersion of Doxorubicin and Metformin HCL micro-particle was prepared just before its incorporation in to a dialysis bag of 12–14 KDa MWCO and start of the dissolution study. Three types of dissolution media, i.e., simulated gastric fluid (pH 1.2), simulated intestinal fluid (pH 6.8) and simulated colonic fluid (pH 7.4) with and without 4% rat cecal content were selected to simulate whole gastrointestinal transit conditions. The release study was conducted firstly in simulated gastric fluid (pH 1.2) for 2 h to mimic the stomach condition. Furthermore, it was done in simulated intestinal fluid (pH 6.8) for the next 3 h followed by in simulated colonic fluid (pH 7.4) for the next 19 h. At the specified time interval, 100  $\mu\text{L}$  aliquot was withdrawn and replaced with the fresh respective buffer solution maintained at  $37^\circ\text{C}$ . The withdrawn samples were filtered through membrane filter with pore size 0.22  $\mu\text{m}$  (MDI, India), diluted suitably and assayed for Doxorubicin and Metformin HCL content (12).

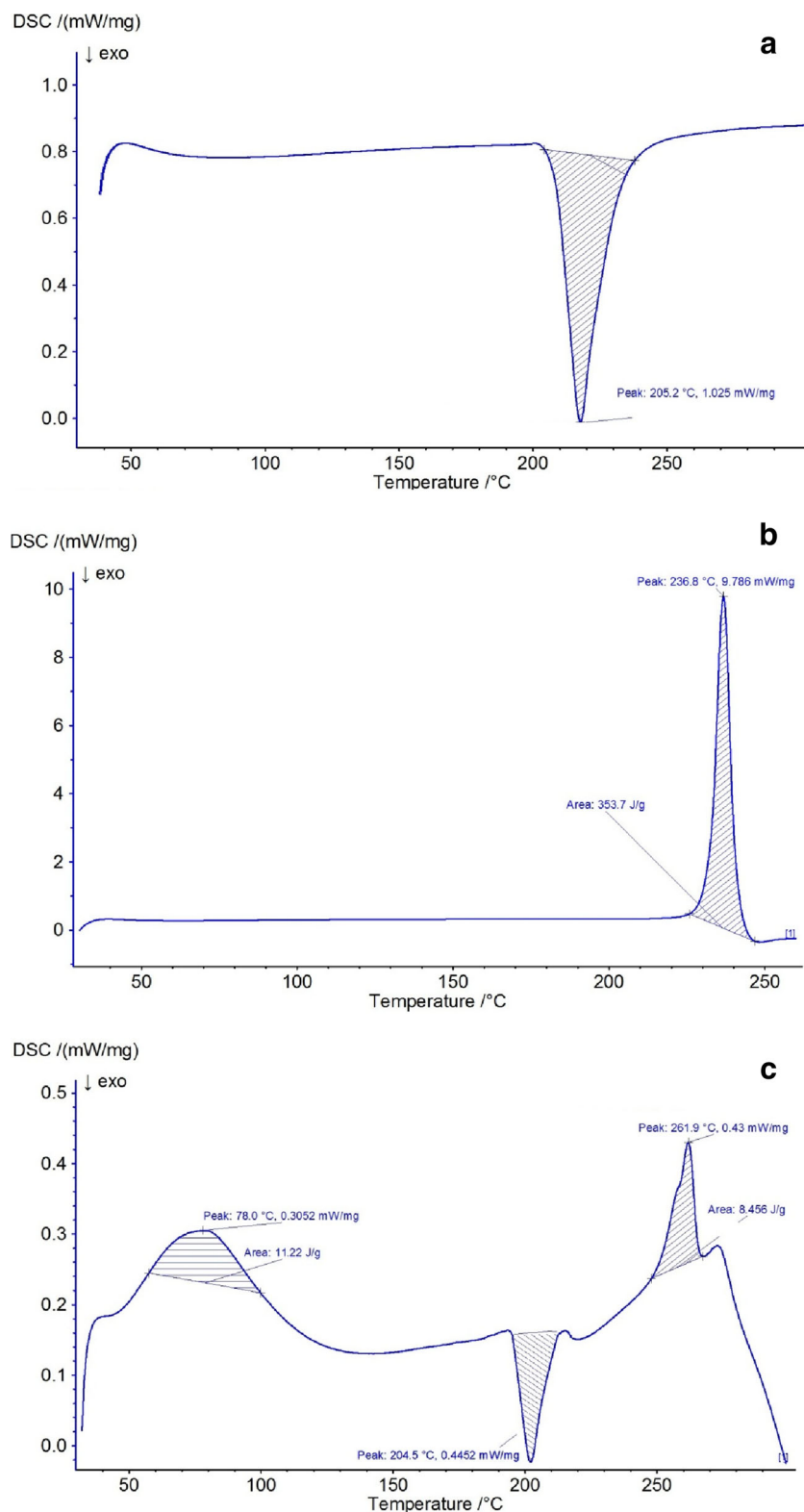
Rat caecal content was added to simulated colonic fluid to ensure the maintenance of identical colonic environment and complete release of the loaded drug. Before the collection of rat caecal content, 1 mL volume of 1% w/v guar gum solution in distilled water was administered orally to the rats that induced enzymes specific for the biodegradation of guar gum during its passage through the colon. This treatment was continued at 2<sup>nd</sup>, 4<sup>th</sup> and 6<sup>th</sup> day. Collection of caecal content was done after anesthetizing rats by diethyl ether. Animals were aseptically dissected and caecal contents were removed. About 4 g caecal content was transferred to 100 mL of simulated colonic fluid (pH 7.4) (12,17).

#### Cell Viability Study

*In vitro* cell viability study was done using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay method against EAC (Ehrlich ascites carcinoma) human epithelial colorectal adenocarcinoma cell lines. These cell lines were purchased from National Center for Cell Sciences (NCCS), Pune, India. EAC cells were seeded in 96-well micro-culture plates at  $1 \times 10^4$  cells/well in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and incubated for 24 h at  $37 \pm 0.5^\circ\text{C}$  in 5%  $\text{CO}_2$  incubator. Tested groups (Doxorubicin, Doxorubicin + Metformin HCL aqueous solution and Doxorubicin + Metformin HCL micro-particles) were used at various concentrations (0, 50, 100 and 200  $\mu\text{g}/\text{mL}$ ) in culture medium and incubated with EAC cells for 24 h. The percentage cell growth inhibition was measured using MTT assay standard protocol (26).

#### Induction of Colon Cancer in Rats and Assessment of Anti-Cancerous Activity

Initially, approximately  $3 \times 10^5$  EAC cells per 0.2 mL PBS suspension were injected subcutaneously to induce tumor in total of eight animals. The tumor sizes were measured by vernier caliper at every 2<sup>nd</sup> day and the tumor



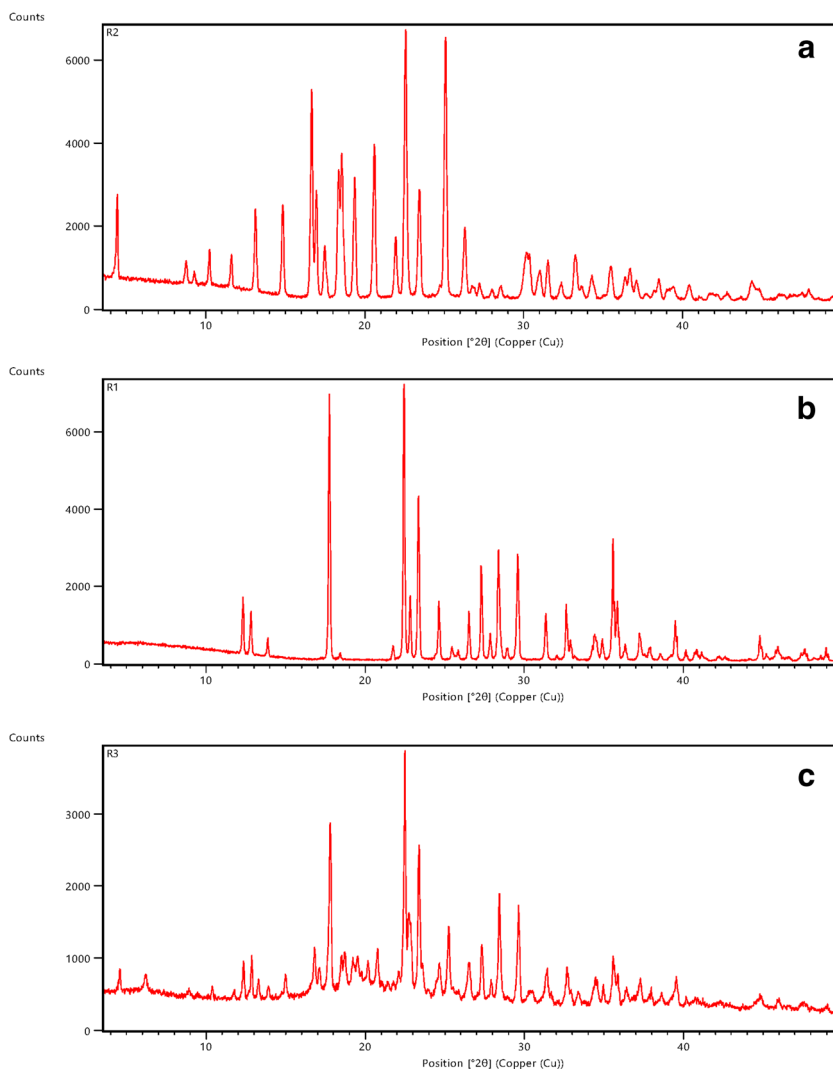
**Fig. 1.** DSC thermogram of Doxorubicin (a), Metformin HCL (b) and guar gum micro-particles of Doxorubicin and Metformin HCL (c)

volumes were calculated using formula given below:

$$V = L \times S^2/2$$

where L is longest length and S is shortest length.

The tumor-bearing rats were selected and euthanized, followed by separation of the developed tumors. Tumors were washed with sterile PBS and cut into size of 2 to 3 mm for the implementation and induction of colon cancer in rats.



**Fig. 2.** XRD pattern of Doxorubicin (a), Metformin HCL (b) and guar gum micro-particles of Doxorubicin and Metformin HCL (c)

Furthermore, 32 rats were divided into 4 groups of eight each namely Disease Control (a), Doxorubicin (4 mg/kgBW)-treated group (b), Doxorubicin (4 mg/kgBW) + Metformin HCL (15 mg/kgBW)-treated group (c) and Doxorubicin (4 mg/kgBW) + Metformin HCL (15 mg/kgBW)-loaded guar gum micro-particles-treated group (d). Cut part of the tumor was implanted into the colon region of the rats of each groups following rectal route and left as such for 14 days. Animals were checked for the induction of colon cancer after surgery in terms of increase in colon diameter or any tumor formation (27). Test samples were administered orally for next 7 days for the treatment of the developed tumors. **Tumor Regression.** Tumor regression analysis was done up to 21 days at 14<sup>th</sup> day and 21<sup>st</sup> day. The estimation of induced tumor was determined by visual and physical inspection in rat colon after dissecting the animal with the help of vernier caliper (28).

**X-ray Imaging of Micro-particles for Targeting Study.** Targeting study of the developed formulation was carried out using a radiographic imaging technique. It

involves the use of radiopaque markers such as barium sulfate, loaded in the micro-particles (at the place of drugs). Barium sulfate micro-particles were used to locate the real time movement and exact position of the micro-particles in rat gastro-intestinal tract. The amount of the barium sulfate was optimized for no effect on the physical characteristics of the optimized formulation (29).

**Histopathology study.** Colon of the animals were harvested at the last day of the study period by sacrificing them 24 h post administration of last dose of the testing formulations. A portion (2 cm) of the colonic specimen from each animal was fixed in 10% formalin, dehydrated, impregnated with the melted wax, molded and cut into 5 $\mu$ m thickness, rehydrated and finally stained with the help of hematoxyline and eosin (H & E dye). Histopathological observations were made in terms of any inflammatory changes like infiltration of the cells, necrotic foci and damages to the tissue structures like lamina propria, villi and intestinal damage to nucleus (22).

### Storage Stability Studies

The prepared guar gum microparticles of Doxorubicin and Metformin HCL were subjected to the storage stability studies. This investigation was carried out as per ICH guidelines at  $5 \pm 2^\circ\text{C}$ ,  $25 \pm 2^\circ\text{C}/60\% \pm 5\% \text{RH}$  and  $40 \pm 2^\circ\text{C}/75 \pm 5\% \text{RH}$ . Significant changes at any condition and time point was observed for the period of 90 days.

### Statistical Analysis

Statistical analysis was performed using SigmaStat software (SPSS). Data were compared between groups using a Student's *t* test. In the case of unequal variances, the Mann–Whitney rank sum test was used. Differences with  $p < 0.05$  were considered significant. All values are presented as mean  $\pm$  SD.

## RESULTS AND DISCUSSION

### Morphological Characteristics and Particle Size of the Prepared Micro-particles

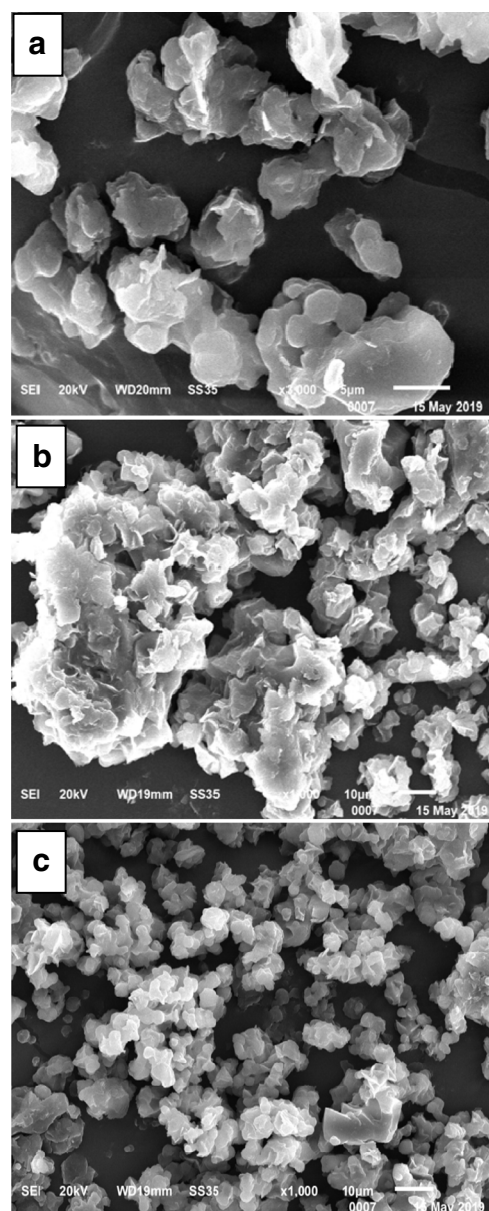
SEM of doxorubicin microparticles, Metformin HCL micro-particles and Doxorubicin and Metformin HCL micro-particles is shown Fig. 3. All types of micro-particles were found to have somewhat spherical shape; uniform size distribution and smooth surface with particle size approximately  $15 \mu\text{m}$  (Fig. 3a–c). This size range of micro-particles could be useful for better colon drug delivery (30).

### Doxorubicin and Metformin HCL–Loaded Guar Gum Micro-particles

Doxorubicin and Metformin HCL–loaded guar gum micro-particles were prepared successfully by mixing Doxorubicin and Metformin HCL micro-particle physically according to their respective oral doses. These micro-particles were characterized for complex formation, drug polymer interaction, drug loading, and entrapment efficiency and evaluated for *in vitro* drug release, *in vitro* efficacy against cancerous EAC cell lines and *in vivo* activity against colon cancer and the results for the same investigations are given hereunder.

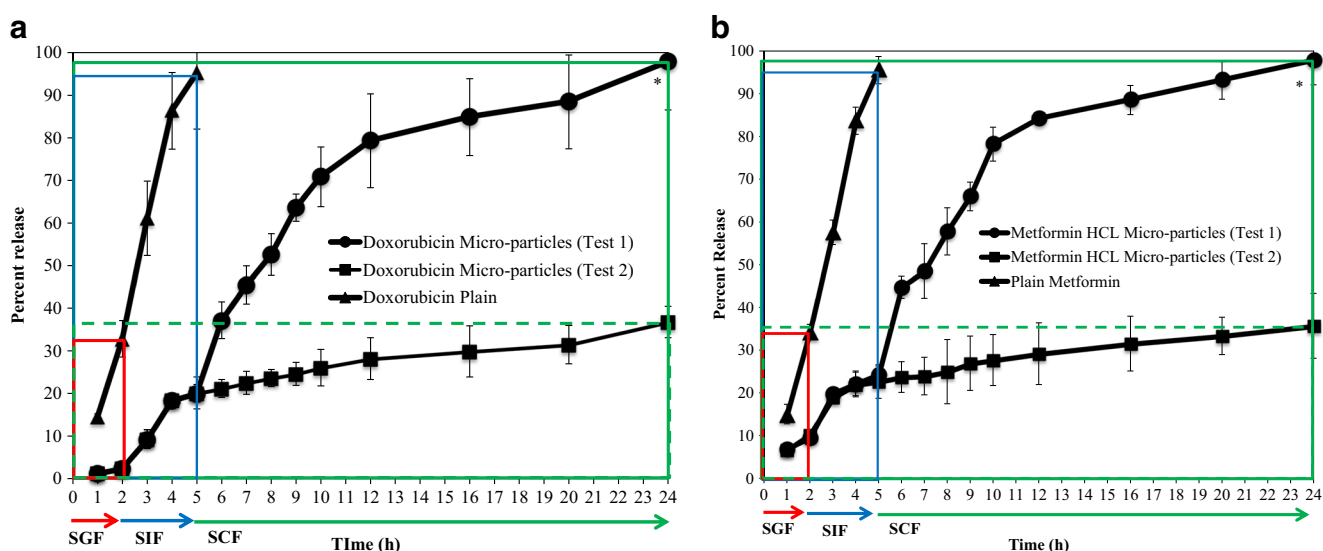
### Differential Scanning Calorimetric

DSC is a thermo-analytical technique, which is used to study the phase transition temperature of drugs and their polymorphic transitions in final formulation. Powdered doxorubicin showed sharp endothermic peak at  $207.3^\circ\text{C}$ , which was found very close to its melting point (Fig. 1a). Pure Metformin HCL showed sharp exothermic peak at  $236.8^\circ\text{C}$  (Fig. 1b), i.e., close to its melting temperature. Thermo-gram of the prepared formulation showed all the three peaks, i.e., for guar gum, doxorubicin and Metformin HCL. Formulation thermo-gram (Fig. 1c) indicates a very broad exothermic peak at  $78.0^\circ\text{C}$ , that is, the characteristic



**Fig. 3.** SEM of Doxorubicin micro-particles (a), Metformin HCL micro-particles (b) and mixture of Doxorubicin and Metformin HCL micro-particles (c)

of guar gum. Second endothermic peak was observed at  $204.4^\circ\text{C}$ , i.e., similar to the endothermic peak of doxorubicin and third exothermic peak that appeared at  $261.9^\circ\text{C}$  indicates slight shift in the melting point of Metformin HCL. Thermo-grams indicate some shift in the peaks especially in the case of Metformin HCL that reflect some degree of physical interaction between Metformin HCL and guar gum. However slight shift in endothermic peak of Doxorubicin showed slight interaction with guar gum. These thermo-grams also indicate good compatibility between drugs and polymer (23).



**Fig. 4.** **a** *In vitro* Doxorubicin release pattern from plain Doxorubicin solution and Doxorubicin-loaded guar gum micro-particles and **b** *in vitro* Metformin HCL release pattern from plain Metformin HCL solution and Metformin HCL-loaded guar gum micro-particles in SGF, SIF and SCF. Test 1 is SCF with rat cecal content, and test 2 is SCF without rat cecal content. Data is presented as mean  $\pm$  SD,  $n = 3$ . \*depict statistically significant difference between test 1 and test 2 ( $p < 0.05$ )

### Physical State of Formulation

XRD graphs of Doxorubicin, Metformin HCL and their micro-particles are shown in Fig. 2. XRD is a phase analysis, which tells about the phase identification of a crystalline material. Doxorubicin showed strong diffraction peaks at  $2\theta$ ;  $16.63^\circ$ ,  $18.53^\circ$ ,  $22.54^\circ$ ,  $25.04^\circ$ ,  $20.5^\circ$ ,  $19.34^\circ$ ,  $23.4^\circ$ ,  $26.2^\circ$ ,  $30.1^\circ$ ,  $31.4^\circ$ ; however, Metformin HCL showed strong diffraction peaks at  $2\theta$ ;  $17.73^\circ$ ,  $22.4^\circ$ ,  $23.3^\circ$ ,  $28.3^\circ$ ,  $29.5^\circ$ ,  $35.5^\circ$ ,  $31.3^\circ$ , which are well supported by the reported literature. The characteristic sharp diffraction peaks with high intensity represent the crystalline nature of both the drugs. Diffraction pattern of Doxorubicin and Metformin HCL-loaded guar gum micro-particles showed peaks at  $2\theta$ ;  $17.7^\circ$ ,  $22.4^\circ$ ,  $23.3^\circ$ ,  $31.3^\circ$ . The intensity of X-ray diffraction pattern of the developed micro-particles was found to be less as compared with that of pure drugs that is attributed to the change in phase of the crystalline drugs in polymer complex with drugs. It also showed the presence of characteristic peak of both the drugs in the XRD of micro-particles suggest no change in chemical properties and presence of physical interaction between drugs and guar gum matrix (31).

### Drug Loading and Entrapment Efficiency

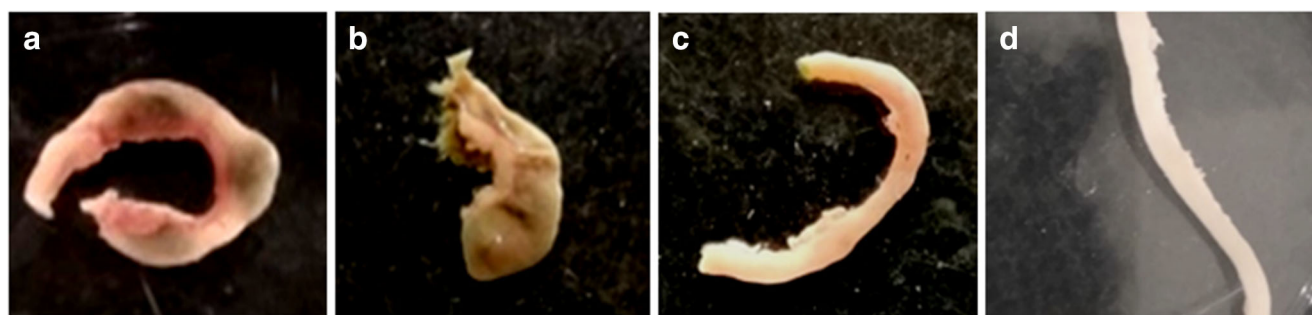
Drug loading in the prepared Doxorubicin and Metformin HCL-loaded micro-particles was found to be 21.65 and 27.17%, respectively. Entrapment efficiency of Doxorubicin and Metformin HCL-loaded micro-particles was found to be 80.2 and 90.3%, respectively, which shows the ability of guar gum in loading drugs and efficient entrapment of both the drugs inside micro-particles when cross linked with glutaraldehyde (21).

### *In Vitro* Release Pattern of Doxorubicin and Metformin HCL from Micro-particles

The Doxorubicin and Metformin HCL release study from the developed micro-particles of guar gum gives an idea about drug release pattern from guar gum micro-particles. This study was done to mimic the whole GI tract as our major goal was to develop colon targeted drug delivery systems of Doxorubicin and Metformin HCL. Prepared micro-particles were subjected to different simulated GI environments and the results reveal the system suitability in colon targeting as major portion of the drugs (about 70%) was found to be

**Table I.** Percent EAC Cell Viability

Concentration ( $\mu\text{g/ml}$ )	Doxorubicin (%)	Doxorubicin + Metformin HCL (%)	Doxorubicin + Metformin HCL micro-particles (%)
Blank	97.73	94.76	90.20
50	81.40	76.52	72.88
100	66.58	42.76	51.33
200	48.9	28.85	32.45



**Fig. 5.** Pictures of size of colon cancer in different groups of animals. **a** Disease, **b** Doxorubicin, **c** Doxorubicin + Metformin HCL and **d** Doxorubicin + Metformin HCL-loaded guar gum micro-particles

released in colonic region. Any oral intake takes at least five hours to reach to the large intestine. Therefore it was required to ensure minimum drug release during first five hours in simulated gastric fluid and simulated intestinal fluid. From the prepared guar gum micro-particles, 20% and 24% release was observed in the case of Doxorubicin and Metformin HCL in stomach and intestinal environment, respectively. It depicts guar gum's ability to restrict drugs release in the physiological environment of stomach and small intestine. This little amount of the drug release during this period might be due to the presence of un-entrapped drug on the surface of the micro-particles or diffusion of drug from outer surface of the guar gum micro-particles (32). Same type of release pattern was observed in SCF also where micro-particles showed only 16 and 13% release of Doxorubicin and Metformin HCL, respectively, in the next 19 h. However, addition of 4% rat caecal content to the SCF modified the release pattern of the micro-particles and we observed significant improvement in drug release from micro-particles. The release of Doxorubicin and Metformin HCL in the SCF with cecal content was observed to be  $97.7 \pm 0.35\%$  and  $97.6 \pm 0.41\%$ , respectively (Fig. 4 a, b). The release of the drugs might be accelerated due to the combined effect of the swelling behavior of guar gum as well as by erosion of guar gum under the influence of colonic enzymes induced after treating rats with aqueous guar gum solution (12).

### ***In Vitro* Viability of EAC Cells**

The cytotoxicity study of the Doxorubicin and Metformin HCL-loaded micro-particles against Doxorubicin and Metformin HCL carried out using MTT assay in EAC cells depicts higher toxicity of Doxorubicin and Metformin HCL at different

concentrations. This combination showed dose dependent increase in cytotoxic potential of Doxorubicin and Metformin HCL as compared with that of Doxorubicin alone. Doxorubicin and Metformin HCL micro-particles also showed good cytotoxic potential, i.e., higher than Doxorubicin alone and lower than Doxorubicin and Metformin HCL solution at the same concentration points. At the highest tested dose (200  $\mu\text{g}/\text{mL}$ ), decreasing pattern of cytotoxic potential of tested groups was found to be in the order of Doxorubicin and Metformin HCL solution > Doxorubicin and Metformin HCL micro-particles > Doxorubicin solution. The higher cytotoxicity in the case of Doxorubicin + Metformin HCL solution (28.85% viability) as with that of Doxorubicin and Metformin HCL micro-particles (32.45% viability), could be due to the direct availability of these drugs around the cells as compared with Doxorubicin and Metformin HCL micro-particles (showed controlled release of drugs). Higher cytotoxicity in the cases of Doxorubicin and Metformin HCL solution as compared with that of as compared with Doxorubicin solution alone (42.9%) could be due to the synergistic effect (7) of Metformin HCL with Doxorubicin (Table I). Moreover, the combined benefits of Doxorubicin and Metformin HCL asserted immense potential of this combination in tumor suppression against colorectal cell lines that can be used as adjuvant on chemotherapy in wide application (33).

### **Anti-Cancerous Effect of Doxorubicin and Metformin HCL Micro-particles**

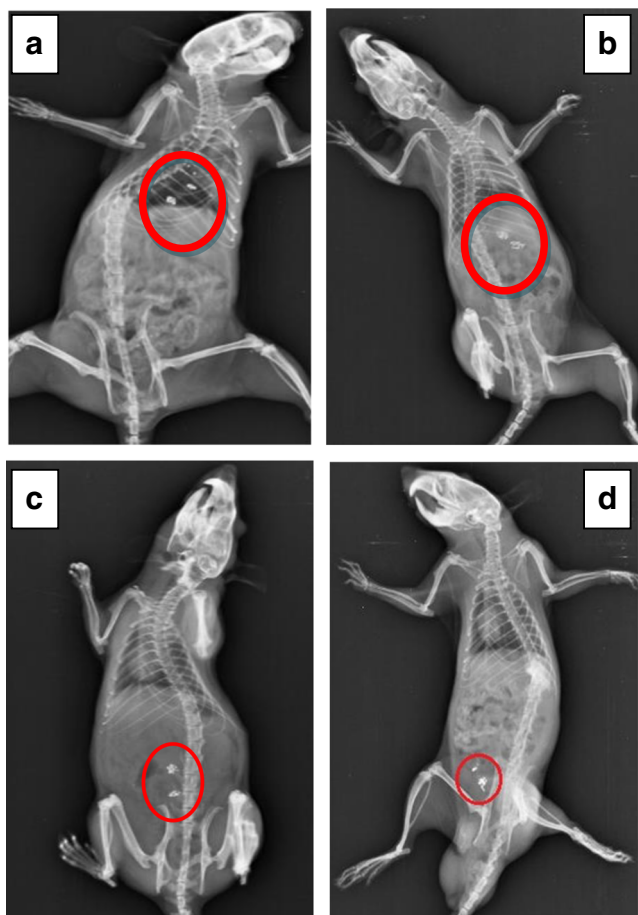
Since Doxorubicin and Metformin HCL combination gave better cytotoxic effect as compared with that of Doxorubicin alone, its *in vivo* effect has to be investigated

**Table II.** Enumeration of Recovery in Tumor Volume (%) After Treating Animals with Doxorubicin Solution, Doxorubicin + Metformin HCL Solution and Doxorubicin + Metformin HCL Micro-particles

S. No.	Group	Tumor volume ( $\text{mm}^3$ )	Recovery in tumor volume (%)
1	Disease control	$4.8 \pm 0.25$	00.00
2	Doxorubicin	$3.89 \pm 0.34^*$	18.95
3	Doxorubicin + Metformin HCL	$3.45 \pm 0.28^*$	28.12
4	Doxorubicin + Metformin HCL micro-particles	$2.15 \pm 0.15^*$	78.20

Data is presented as mean  $\pm$  SD,  $n = 3$ . \*depict statistically significant difference between disease control and test control groups ( $p < 0.05$ ).





**Fig. 6.** X-ray imaging rats by BaSO<sub>2</sub>-loaded micro-particles. X-ray images of rats after 2<sup>nd</sup> hour (a), 4<sup>th</sup> hour (b), 6<sup>th</sup> hour (c) and 8<sup>th</sup> hour (d) depict the intactness of the micro-particles at different time points

against the prepared micro-particles. Micro-particles were prepared to target Doxorubicin and Metformin HCL combination specifically to the colon. *In vivo* investigation suggests utility of the developed system in targeting these drugs to colon and in the improving anti-tumor efficacy. This investigation was carried out on the basis of various parameters and the results for the same are as follows.

#### Tumor Regression

Tumor regression studies were performed by excising tumors on 21<sup>st</sup> day of the study period. Pictures of the excised colon with tumors are depicted in Fig. 5 a–d. Results obtained from the tumor regression studies indicated considerable differences in the tumor volume of different treatment groups. The tumor volume of different groups is given in Table II along with percent recovery in volume after treatment. It has been observed that the plain Doxorubicin resulted in a decrease in tumor volume, which is due to its anticancer potential. The group treated with plain Doxorubicin and Metformin HCL (28.12%) resulted good recovery in tumor volume as compared with that of Doxorubicin alone (18.95%). However, percent

recoveries in tumor volume of Doxorubicin and Doxorubicin + Metformin HCL solution-treated groups were barely noticeable because these gave very less recovery even after 21 days. This could be due to the higher availability of drugs in upper GIT as compared with that of lower GI tract. The group treated with Doxorubicin and Metformin HCL micro-particles showed increased therapeutic efficacy against colon cancer and showed about 78.12% recovery in tumor volume. This might be due to the site specificity/targetability of the delivered drugs following oral route of administration.

#### Targetability of the Developed Micro-particles

X-ray imaging showed targeting ability of guar gum to colon. Figure 6 depicts successful targeting of micro-particles to colon by X-ray pictures of rats at different time interval following oral administration. Minimal degradation of guar gum micro-particles was observed in stomach and small intestinal regions. Bacterial dependent guar gum release of the drugs ensures colon specific drug delivery. X-ray images prove the target ability of the developed guar gum micro-particles that make it feasible option for specific for the delivery of drugs to the colon (34).

#### Histopathological Features

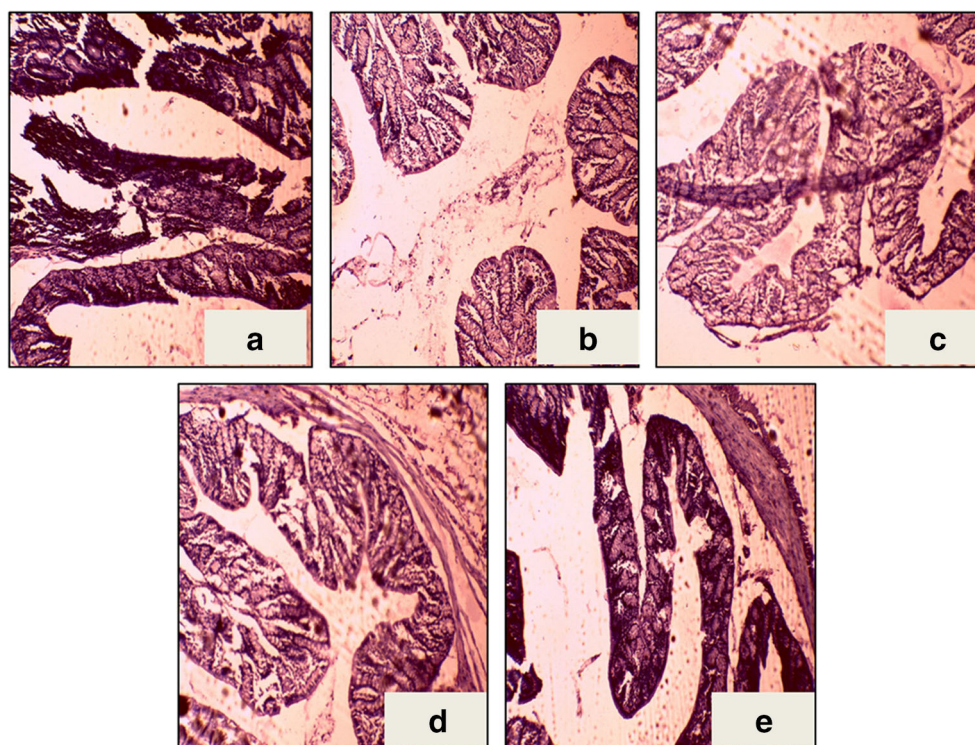
The results of the histopathology studies of the group treated with plain Doxorubicin and Metformin HCL and the group treated with micro-particles are shown in Fig. 7. Histopathology features of diseased group confirm the presence of tumor cells. Presence of cancerous cells in plain Doxorubicin and Metformin HCL combination-treated group is comparatively higher as compared with the group treated with the Doxorubicin and Metformin HCL-loaded micro-particles. A significant reduction in the violet color zone can be observed in micro-particles-treated group as compared with the plain combination of Doxorubicin and Metformin HCL indicating high anti-cancer potential of the prepared formulation. Higher efficacy of the prepared micro-particles over the conventional administration might be due to the site specific delivery of the drugs in contrast to their combination in solution form (22).

#### Storage Stability of the Developed Micro-particles

Table III illustrates the physical storage stability study of the developed microparticles. It was observed stable at 25 and 5°C. The consistency of the micro-particles was maintained for the whole period of investigations. However, above 25°C or at 40°C, these particles showed some degree of instability in long term storage.

#### CONCLUSION

This investigation portrays the importance of Doxorubicin and Metformin HCL combination in reducing the viability of EAC cells. Preclinical investigations reveal the importance of guar gum-based targeted approach for the



**Fig. 7.** Histopathology images of rat colon of different treatment groups. Histopathology images of normal colon (a), diseased colon (b), Doxorubicin-treated colon (c), Doxorubicin + Metformin HCL-treated colon (d) and Doxorubicin+ Metformin HCL micro-particles-treated colon (e)

specific delivery of these drugs to the colonic site for better effect. The prepared micro-particles of guar gum were able to provide protection to the drug in the hostile environment of upper gastrointestinal tract and restricted release in small intestine. The *in vitro* drug release studies revealed the role of enzymes in the erosion of guar gum and hence ensured maximum release in simulated colonic fluid in the presence of rat cecal content. *In vitro* MTT assay reflects utility of Doxorubicin and Metformin HCL combination over Doxorubicin alone and *in vivo* anti-cancerous activity reflects utility of Doxorubicin and Metformin HCL-loaded guar gum micro-particles over Doxorubicin and Metformin HCL combination. The developed micro-particles with good stability were observed to be able to target Doxorubicin

showed immense potential to work as tool to utilize the synergistic effect of this combination.

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**Table III.** Storage Stability of Guar Gum Micro-particles of Doxorubicin and Metformin HCL as per ICH Guidelines

Storage condition	Particle size ( $\mu\text{m}$ )			Entrapment efficiency (%)					
				Doxorubicin micro-particles			Metformin HCL micro-particles		
	0 <sup>th</sup> day	30 <sup>th</sup> day	90 <sup>th</sup> day	0 <sup>th</sup> day	30 <sup>th</sup> day	90 <sup>th</sup> day	0 <sup>th</sup> day	30 <sup>th</sup> day	90 <sup>th</sup> day
5 $\pm$ 2°C	15.2 $\pm$ 1.10	17.5 $\pm$ 1.23	18.2 $\pm$ 1.30	80.2 $\pm$ 7.5	79.8 $\pm$ 4.9	79.2 $\pm$ 7.6	90.3 $\pm$ 7.8	89.4 $\pm$ 5.78	88.9 $\pm$ 9.50
25 $\pm$ 2°C/60% RH	15.2 $\pm$ 1.10	18.5 $\pm$ 0.9	23.5 $\pm$ 2.10*	80.2 $\pm$ 7.5	78.6 $\pm$ 6.2	75.5 $\pm$ 6.5	90.3 $\pm$ 7.8	88.6 $\pm$ 6.00	83.3 $\pm$ 6.40*
40 $\pm$ 2°C/75%RH	15.2 $\pm$ 1.10	18.7 $\pm$ 0.85	34.5 $\pm$ 4.50*	80.2 $\pm$ 7.5	75.8 $\pm$ 5.3	68.8 $\pm$ 4.5*	90.3 $\pm$ 7.8	86.3 $\pm$ 4.80	78.3 $\pm$ 3.80*

Data is presented as mean  $\pm$  SD,  $n = 3$ . \*depict statistically significant difference between the values at 0<sup>th</sup> day and 90<sup>th</sup> day ( $p < 0.05$ ). and Metformin HCL combination specifically to colon and

## COMPLIANCE WITH ETHICAL STANDARDS

**Conflict of Interest** The authors declare that they have no conflict of interest.

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