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Improving the therapeutic efficiency of ginger extract for treatment of colon cancer using a suitably designed multiparticulate system

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Abstract

Ginger extract (GE), a potential natural anticancer agent, has compromised therapeutic utilization due to poor bioavailability and physicochemical properties. Present study aimed at assigning GE with a pharmaceutical couture so as to improve its biopharmaceutical performance by monitoring its localized (though prolonged) delivery in the distal parts of gastrointestinal tract for the treatment of colon cancer. Alginate beads entrapping 85.9 ± 1.78% GE were subjected to Eudragit S100 coating. Latter is insoluble at acidic and near neutral (6.8) pH of stomach and upper part of small intestine and it led to 50% retardation (upto 12 h) in release of GE. However, it was solubilised at pH > 7.0 resulting in colon targeted system. Developed beads were free flowing, showed a particle size of 0.9 ± 0.006 mm and super class-II release controlled by swelling and polymer relaxation. Preclinical evaluation using 1,2-dimethylhydrazine-induced colon cancer, in male Wistar rats, in terms of histopathology, oxidative stress, mitochondrial complex activity, β-glucuronidase and ammonia concentration determinations indicated GE loaded beads (50 mg/kg) to be significantly better ($p < 0.05$) than free GE. Highlight of the study was that GE loaded coated alginate beads were administered after the induction of colon cancer and significant recession of the cancers was observed after 4 weeks of treatment.

Keywords

Alginate beads, colon cancer, dimethylhydrazine, Eudragit S100, ginger extract

History

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Introduction

The unique physiological characteristics of colon such as the presence of microflora, acidic environment (pH 6.0–6.4 in the proximal colon), high luminal pressure and small effective surface area available for absorption [1], can be explored for targeting drugs to treat diseases of the colon. Minimizing the absorption of drugs from stomach and small intestine ensure larger amounts to reach the large intestine eliciting an effective pharmacological response with a reduced incidence of side effects [2].

An intricate link has been established between chronic inflammation and cancer. Various epidemiological studies indicate that dietary intervention with antioxidant/anti-inflammatory agents prompts an interest in food components that tend to attenuate cancer risk significantly [3]. Ginger (*Zingiber officinale* Roscoe, Zingiberaceae), rich in polyphenolic compounds, like [6]-gingerol, shogaols, paradols and zingerone, is high in antioxidant value with an ability to scavenge a number of free radicals and protect cell membrane lipids from oxidation [4]. What sets ginger apart is its unique property of inhibiting not only prostaglandin (PG) synthesis but also leukotriene (LT) synthesis. Such dual inhibitors of cyclooxygenase (COX) and lypoxygenase (LOX) are more effective and have fewer gastrointestinal side effects than pure COX inhibitors. That is why even high doses of ginger extract (GE) do not produce the side effects often observed with non-selective COX inhibitors [4]. It was found that ginger preparation inhibits COX-2 more than COX-1. However, remarkable pharmacological activities of ginger or 6-gingerol are compromised by its poor pharmacokinetic properties. 6-Gingerol is cleared very rapidly from plasma with a short terminal half life of 7.23 min and a total body clearance of 16.8 ml/min/kg after intravenous administration [5]. After oral administration, 6-gingerol is rapidly absorbed into the plasma and the maximal concentration (4.23 μg/ml) is reached within 10 min of oral dosing [6].

Present study was thus undertaken with an aim to modify and improve the pharmacokinetic behaviour of GE, using a suitable delivery system and offer it as a promising therapeutic option to the oncologists for the treatment (and not only prevention) of colon cancers. We have earlier reported on the use of calcium alginate floating beads of GE for improved and sustained release in the stomach area to control gastric ulcers [7]. Presently, we propose to extend the concept of using alginate beads suitably coated with Eudragit S100 for colon (mainly its distal part) targeted release. Eudragit S100 dissolves at pH > 7.0 (i.e. colonic pH), thus avoiding the release of entrapped GE in the upper gastrointestinal tract (GIT) including the proximal part of colon because of the prevalence of slightly acidic environment (pH 6.0–6.4) at this site owing to degradation of poly- and

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oligosaccharides. It may be noted here that use of alginate beads suitably coated with Eudragit S100 to achieve colon targeting, though reported frequently for incorporation of active pharmaceutical ingredients (APIs), has never been explored for natural molecules and extracts especially GE. The concept is novel and has not been reported for improved GE delivery.

Multiparticulate systems (MPSs) are preferred because single unit systems show disadvantages of unintentional disintegration and compromised bioavailability. Small particle size of MPS allows easy passage through the GIT making it a reliable system, with less inter and intra-subject variability [8]. Calcium alginate beads formed on gelation of sodium alginate with calcium chloride is one of the most widely studied MPS. Properties like biocompatibility, bioadhesiveness, pH sensitivity and mild gelation make this system suitable for oral delivery [3]. A localized slow release and an extended stay in colon will improve therapeutics of GE which is otherwise quickly absorbed into and lost from systemic circulation due to fast elimination. A slow diffusion and extended release will ensure a significant effect.

Protective role of dietary antioxidants, in preclinical studies, is usually indicated as a pretreatment therapy. Latter, it has no clinical significance in the sense that administration of the agent is indicated prior to disease occurrence, which is rare and impractical. The intent of the present study is strengthening of the therapeutic armamentarium for the treatment of cancers after their occurrence using natural extracts. New drug discovery is a long, tedious, time consuming and a very costly (both in terms of money and manpower) process. Hence revising the claims of traditional medicine, supplemented with proven *in vitro* and *in vivo* studies, establishing a suitable mechanism of action of these agents should be a wise and rational approach. However, various factors namely high recommended dose, poor pharmacodynamic profile, adverse effects and poor stability hinder the translation of these agents to therapeutics. It is at this juncture that a formulation scientist needs to step in and bridge the gap.

The present study very aptly describes development of an effective Eudragit S100-coated calcium alginate beads and gives evidence not only of its optimal colon targeting but also establishes its effective and prolonged release in the colon. The formulation was evaluated in terms of its protective effect against colon cancer, post-induction, in terms of oxidative stress, mitochondrial enzyme complex activity, β glucuronidase and ammonia concentration determination and colon cancer-induced histopathological changes. All these are well-established markers to determine the occurrence and extent of cancers in general and colon cancer in particular. Study may thus be considered as a prototype, which establishes the importance of amalgamating rational formulation design with observational data from the folklore complemented with scientific and precise *in vitro* and *in vivo* findings of the molecular biologist, pharmacologist and biochemists to bring a new class of safe, cheap and effective curatives to the market. We passionately and strongly feel the need to elevate the status of those natural extracts and molecules from preventive dietary supplements to curative therapeutics.

Methods

Materials

GE (16% gingerol and 6% zingiberene) was procured as a gift sample from Nisarga Biotech, Satara, India. It is prepared under supercritical CO_2 extraction at 300 Bar and 39 °C; and the content of gingerol was 16% as determined by high performance liquid chromotography. Sodium alginate, calcium chloride, polyethylene glycol (PEG 400) and methanol were purchased from S.D. Fine Chem. Ltd., Mumbai, India, and were of analytical reagent (AR) grade. All other chemicals or reagents used in the study were of AR or guaranteed reagent (GR) grade. Eudragit S100 was received as a gift sample from Evonik Roehm Pharma Polymers batch no. B071003084. All *in vitro* and *in vivo* experiments were done in the labs of University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh, India in the year 2011.

Revalidation of spectrophotometric method of analysis for GE in different solvents

Spectrophotometric method of analysis of GE has already been validated and reported by us [7]. Calibration curves (λ_{max} 281 nm) of GE were however constructed presently in three buffer solutions pH 1.2-methanol (9:1), pH 6.8-methanol (9:1) and pH 7.4-methanol (9:1), using UV spectrophotometer, and analytical method was validated with respect to linearity, accuracy and precision.

In vitro antioxidant activity of GE

Four different test systems 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [9], superoxide anion scavenging [10], hydrogen peroxide scavenging [11] and nitric oxide scavenging [12] were used for determining antioxidant properties of GE against vitamin C as standard.

Preparation of GE beads

Calcium alginate beads were prepared by orifice ion gelation method [7,13]. Briefly, 3 g of GE was dissolved in 5 ml PEG 400 to obtain a clear solution which was then dispersed in an aqueous sodium alginate solution (3% w/v). The mixture (after degassing for 20–30 min in bath sonicator) was dropped through a 26 G syringe needle into 1% w/v calcium chloride solution under magnetic stirring at 60 rpm. The stirring was continued for 1 h after completion of the addition to improve mechanical strength of the suspended beads, after which the beads were separated, washed initially with alcohol and subsequently with distilled water followed by overnight freeze drying of the beads at −40 °C. The product was lyophilized further for 6 h at −70 °C. Lyophilized beads were coated with Eudragit S100 upto two levels (10% and 15% weight gain) using a fluid bed coater (Plam Glatt, Germany) and by spraying the coating solution continuously from the bottom of the bed to prewarmed (upto 35 °C inlet and 30 °C product temperature) fluidized beads.

Determination of drug entrapment efficiency (DEE) of GE beads

Accurately weighed GE beads (50 mg) were crushed in a mortar and 15 ml methanol was added to them. The mixture

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was transferred to a tube, mixed thoroughly on a vortex, centrifuged and the supernatant collected, suitably diluted with methanol and analysed spectrophotometrically against methanol as a blank. DEE was calculated according to the following equation:

$$\%\text{DEE} = \frac{\text{Actual drug content in beads}}{\text{Theoretical drug content}} \times 100$$

The experiment was repeated for at least six batches of GE beads prepared on separate occasions, to validate the method. A scale up batch was prepared for subsequent characterizations and *in vivo* evaluation.

In vitro release of GE from developed beads

Drug release studies were carried out using a USP Dissolution Apparatus (Apparatus I, 100 rpm, 37 °C). The coated calcium alginate beads were tested for drug release for 2 h in 0.1 M HCl:methanol (9:1; 500 ml). This was done to simulate gastric conditions and the study was done for 2 h as the average gastric emptying time is about 2 h. Then the dissolution medium was replaced with pH 6.8 phosphate buffer:methanol (9:1; 500 ml) and tested for drug release for 3 h, as the average time taken for transit through small intestinal is about 3 h. pH 7.4, phosphate buffer:methanol (9:1; 500 ml) was used finally to test for the drug release upto 24 h. Samples (5 ml) were withdrawn at predetermined time intervals and replaced with same volume of the similar fresh medium. Amount of GE released was analysed spectrophotometrically at 281 nm. Release pattern obtained with coated beads (both 10% and 15% weight gain) were compared with those for uncoated beads to check for the efficiency of coating. *In vitro* data were evaluated both for the order and mechanism of release.

Swelling studies

The swelling properties of the Eudragit-coated GE beads were determined *in vitro* at a pH ranging from pH 1.2 (for 2 h), through 6.8 (2–5 h), upto pH 7.4 (5 h onwards) using suitable buffer solutions. Weight of 30 completely dry beads was noted, and were allowed to swell at 37 °C by placing them in a small beaker containing 100 ml of respective buffer solutions. Beads were shifted to beakers containing subsequent pH buffers at specified time intervals. After fixed time points, swollen beads were reweighed to record the increase in weight. The magnitude of swelling was presented by the ratio of the mean weight of swollen beads to the mean weight of the dry beads, at the start of the test.

$$\text{Swelling ratio} = \frac{\left\{\begin{array}{c}(\text{Mean weight at time } t\\ -\text{Mean initial weight})\end{array}\right\}}{\text{Mean initial weight}} \times 100$$

Micromeritics

The diameter of calcium alginate beads was determined using particle size analyser (Malvern Instruments Limited, Malvern, UK). Flow properties of the beads were investigated by measuring the angle of repose of drug loaded beads using fixed-base cone method. Briefly, the beads were allowed to fall freely through a funnel, fixed at 1 cm above the horizontal flat surface, until the apex of the conical pile just touched the tip of the funnel. Height and diameter of the cone were measured and angle of repose was calculated using the formula:

$$\text{Angle of Repose } (\theta) = \tan^{-1} \frac{\text{Cone height}}{\left\{\begin{array}{c}\text{Radius of circular base formed}\\ \text{by beads on ground}\end{array}\right\}}$$

Each experiment was carried out in triplicate.

The bulk and tap densities were determined using a 10 ml graduated cylinder as a measure of packability of the beads. The sample contained in the measuring cylinder was tapped mechanically (25 times). The initial bulk volume and final tap volume were noted and the respective densities were calculated from these values.

Hauser ratio of beads was determined by comparing the tap density to the bulk density by using the equation:

$$\text{Hauser ratio} = \text{Tap density}/\text{Bulk density}$$

In vivo evaluation in 1,2-dimethylhydrazine-induced colon cancer model

Induction of colon cancer

Male Wistar rats, not more than 250 g, bred in the Central Animal House, Panjab University, Chandigarh, India, were used. Animals of each group (six animals) were caged together and kept under natural light/dark cycle, were given food and water *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee, Panjab University, Chandigarh, India. Animals were divided into four groups; each group consisting of six animals. Group I (naive control) consisted of animals receiving no treatment and served as a negative control. Animals of group II–IV received a subcutaneous injection of 1,2-dimethylhydrazine (DMH) (30 mg/kg body weight in 1 mM EDTA, pH 6.5), once a week, for 18 weeks (establishment of colon cancer was confirmed at this time by sacrificing three animals and observing for development of aberrant crypt foci). Group II (DMH group) served as the positive control group (receiving no treatment); group III (free GE group) received 50 mg/kg free GE and group IV (GE beads) received beads equivalent to 50 mg/kg GE for 1 month starting from 18th week (of DMH treatment) onwards. DMH treatment was also continued during this time. A total of 22 weeks of DMH treatment was given to all the rats of groups II–IV.

After a treatment of 4 weeks, all the animals were sacrificed by cervical dislocation and their distal part of colon (last 4 cm) was separated. Part of the colon was fixed with formaldehyde for histopathology studies. Remaining colon was homogenized in 0.1 M phosphate buffer for estimating oxidative stress markers (lipid peroxide [LPO], superoxide dismutase [SOD], nitrite, catalase), β glucuronidase and ammonia levels and homogenizing buffer with ethylene glycol tetraacetic acid (EGTA) for estimation of various mitochondrial complexes.

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Histopathological examination

For histopathological examination of aberrant crypts and foci, colon was opened and quickly fixed in buffered formalin solution.

Biochemical analysis of colonic homogenates

Removed colons were rinsed with ice cold saline and weighted. A 10% w/v colon homogenate was prepared in 0.1 M phosphate-buffered saline (pH 7.4) which was refrigerated and used for lipid peroxidation [14], catalase [15], SOD assay (MnSOD and CuZnSOD) [9], nitrite [16] and protein estimation [17].

Mitochondrial enzyme complex estimation

Rat colon mitochondria were isolated [18]. Mitochondrial suspension in isolation buffer without EGTA was further used for estimation of complex I [19], II [20] and IV [21].

β glucuronidase and ammonia determination

Colon homogenates in phosphate buffer were centrifuged at $500 \times g$ for 3 min to remove debris [22]. The supernatant was decanted and diluted with potassium phosphate buffer and used for enzyme analysis. Assay of β-glucuronidase was carried out at 37 °C. Each sample was incubated with *p*-nitrophenyl-β-D-glucopyranoside for β-glucuronidase. After 2 h the reaction mixture was analysed spectrophotometrically at 402 nm to measure the released *p*-nitrophenol. Ammonia concentration in colon homogenates was determined [23]. Treatment with phenol nitroprusside and alkaline hypochlorite resulted in the development of a blue colour which was measured at 630 nm. Ammonia concentration was determined from standard reference curve using different concentrations of ammonium chloride and the results are expressed as µmol per gram of colon content.

Statistical analysis

The raw data obtained from *in vitro* release study are expressed as mean ± standard deviation (SD). The *in vivo* results are expressed as mean ± standard error of mean (SEM). The intergroup variation was measured by one-way analysis of variance (ANOVA) followed by Dunnett test. Statistical significance was considered at $p < 0.05$.

Results

Revalidation of spectrophotometric method of analysis for GE

Linearity range for GE in all the buffer solvent systems was found to be 10–100 µg/ml ($r^2 = 0.999$) and accuracy and precision were found satisfactory.

In-vitro *antioxidant activity of GE*

Table 1 shows the antioxidant potential of GE determined by us against a series of free radicals generated *in vitro*. All the four tests indicate that GE possesses good antioxidant properties when compared with vitamin C. It was found to show most effective nitric oxide scavenging effect, closely followed by super oxide anion and hydrogen peroxide scavenging.

Table 1. IC_{50} values of ginger extract and vitamin C.

	IC_{50} values ($n = 6$)	
Assay method	Ginger extract (µg)	Vit. C (µg)
DPPH assay method	291.50 ± 2.91	6.69 ± 1.65
Super oxide anion	6.49 ± 0.65	18.41 ± 2.75
Nitric oxide scavenging	2.52 ± 1.05	–
H_2O_2 scavenging	24.45 ± 1.45	26.08 ± 4.08

Table 2. Various formulation parameters for G1 and G2[a].

Formulation code	Sodium alginate (%w/v)	Calcium chloride (%w/v)	GE (%w/v)	% Entrapment (GE) ($n = 3$)
G1	3	1	3	85.9 ± 1.78
G2	3	1	1	80.3 ± 1.53

[a]% entrapment of GE for G1 and G2 is significantly ($p < 0.05$) different.

Drug entrapment

Two different loading concentrations of GE, G1 (3% w/v) and G2 (1% w/v) were used to prepare the beads. Interestingly the entrapment efficiency reduced significantly ($p < 0.05$) from 85.9 ± 1.78% to 80.3 ± 1.53% (Table 2) when the loading amount of GE was decreased from 3% w/v to 1% w/v. Therefore, we proceeded with formulation G1 which was then coated suitably to result in a colon targeted system.

In vitro release: selection of suitable coating

Release of GE from formulated calcium alginate beads was performed successively in different media, pH 1.2 (0.1 N HCl) for 2 h, followed by pH 6.8, phosphate buffer (0.2 M) for 3 h and pH 7.4, phosphate buffer (0.2 M) till 24 h. GE being slightly soluble in water (0.69 ± 0.03 mg/ml) [7] showed very poor solubility in the buffer medias. Hence, 10% methanol was added to all the release media to improve the solubility of GE in respective media and to maintain sink conditions.

Uncoated beads owing to the entrapment of GE in alginate matrix in itself restrain the release of GE in the upper GIT (with <4% GE release in 5 h; Figure 1). However, at subsequent times GE is released very quickly in pH 7.4 phosphate buffer with >71% release within 12 h. Since we wish to target the distal part of colon due to the prominence of tumors in this part [24], hence these beads were sequentially coated with Eudragit S100, first upto 10% weight gain, which significantly slowed the release of GE in comparison to the uncoated beads (25%, 40% and 71% release for uncoated beads and 17%, 25% and 51% for coated beads with 10% weight gain at 8, 10 and 12 h, respectively). The retardation was however not sufficient, considering that 51% GE was still released at 12 h. Hence, the coating was further extended to 15% weight gain. Now the release was sufficiently and significantly ($p \leq 0.05$) retarded even at 12 h, from 71% in uncoated beads, to just 37% in coated beads with 15% weight gain. An almost 50% retardation was thus achieved with respect to uncoated and 28% in comparison to coated beads with 10% weight gain, confirming the development of a desired colon targeted system (Figure 1). Therefore, 15%

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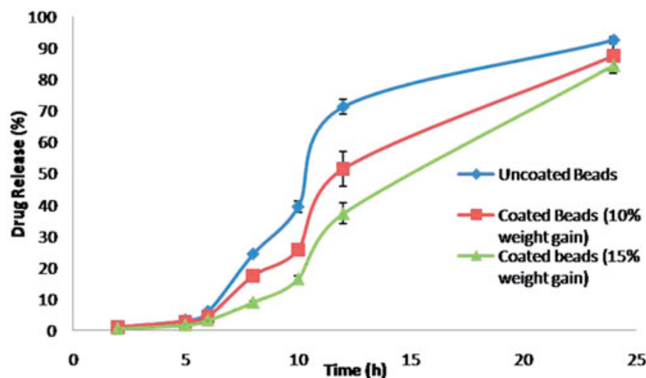

Figure 1. *In vitro* release of GE from coated beads (10% and 15%) and uncoated beads.

Table 4. Comparison of swelling ratio of coated and uncoated GE beads[a].

pH of media	Time (h)	Swelling ratio	
		Coated beads	Uncoated beads
1.2	0	0.019 ± 0.006	0.020 ± 0.009
	2	0.045 ± 0.010	0.309 ± 0.049
6.8	4	0.233 ± 0.063	0.712 ± 0.094
7.4	6	0.978 ± 0.102	2.402 ± 0.172
	8	1.890 ± 0.151	–
	24	–	–

[a]All values for coated and uncoated beads are significantly ($p < 0.05$) different. ''–'' beads lost their integrity.

Table 3. r^2 and n values for uncoated and coated GE beads during the *in vitro* release studies.

pH	Zero order [r^2]		First order [r^2]		Higuchi model [r^2]		Kosmeyer–Peppas [r^2]		n	
	UC	C	UC	C	UC	C	UC	C	UC	C
1.2	0.958	0.959	0.894	0.801	0.801	0.928	0.947	0.978	1.074	0.523
6.8	0.950	0.987	0.919	0.997	0.945	0.994	0.954	0.981	1.220	1.512
7.4	0.834	0.983	0.937	0.990	0.910	0.978	0.929	0.937	1.859	1.357

UC: uncoated; C: coated.

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weight gain coating of GE alginate beads with Eudragit S100 was selected as final formulation.

The *in vitro* dissolution data for uncoated and coated beads were tested in four kinetic models (zero, first, Higuchi and Kosmeyer Peppas) and their r^2 values were compared. In case of uncoated GE beads the release was zero order at pH 1.2 (0.958) and pH 6.8 (0.950), but changed to first order (0.937) after 5 h and in pH 7.4 buffer, while for coated GE beads the zero order release at pH 1.2 (0.957) became first order at pH 6.8 (0.997) and pH 7.4 (0.990). Kosmeyer Peppas release exponent (n) revealed that except for coated GE beads at pH 1.2 with $n = 0.523$ (indicating non-fickian release), the value of diffusion coefficient (n) was >0.82 indicating the drug release from the beads to be super class II, i.e. controlled by the swelling and relaxation of the polymer (Table 3).

Swelling study

Uncoated beads showed very low swelling ratio in acidic pH (Table 4), however, the beads swelled considerably when the pH was increased to 6.8 and later to 7.4. Coating of GE beads with Eudragit S100 ensured better resistance to swelling and drug release, in the upper part of GIT, corresponding to pH 1.2 and 6.8 buffers. This will ensure the release of drug only in the colon as depicted clearly in Figure 2.

Uncoated GE beads tend to release the drug even before the ileocecal junction; whereas the coated beads maintained their integrity and are observed intact till the ileocecal junction after 8 h of peroral administration.

Micromeritics

The average particle size of uncoated and coated GE beads was 746.96 and 895.36 µm, respectively (Table 5). A significant increase in the particle size ($p < 0.05$) was observed after coating with Eudragit S100. The rheological parameters like angle of repose, bulk density and tap density are important to confirm better flow and packaging properties [25].

Both coated and uncoated beads showed excellent flowability represented in terms of an angle of repose <40° (Table 5). Bulk and tap density of beads showed good acceptable range which in turn indicated good packability. This was also supported by the Hauser ratio.

In vivo results

Histopathological evaluation

Histopathological study of gastric mucosa was performed after staining with haematoxylin and eosin (Figure 3). Group I (naive control) showed normal healthy crypts with the basal mucosal glands maintaining their identity. Group II (DMH group) showed tumors projecting into the lumen with insert clearly showing cancerous cells with distorted cell nucleus (Figure 3B). Group III (free GE group) shows presence of excessive lymphoid tissue with the insert highlighting presences of cancerous cells infiltrating into muscle layer. Presence of lymphoid follicles is the normal feature of colon but the lymphoid tissue in this group shows abnormal growth. Group IV (GE beads) again shows a large lymphoid follicle with insert showing precancerous atypical nuclei.

Biochemical analysis

We determined LPO (Figure 4A), catalase (Figure 4B), nitrite (Figure 4C) and SOD (both CuZnSOD and MnSOD; Figure 4D) of the colonic homogenates, of DMH induced and free GE or coated GE beads treated groups and DMH induced cancer control groups, as the markers of oxidative stress and compared with the naive control values. Results suggest that reactive oxygen species (ROS) could be one of the important

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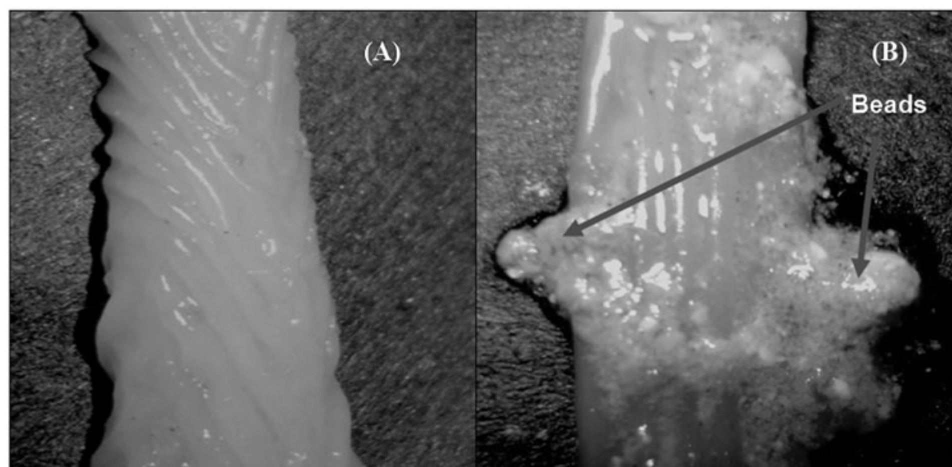

Figure 2. Presence of beads at ileoceal junction after 8 h of oral administration of respective beads to rats. (A) Uncoated beads fail to reach the ileocecal junction and (B) intact beads (coated) are observed at the ileocecal junction.

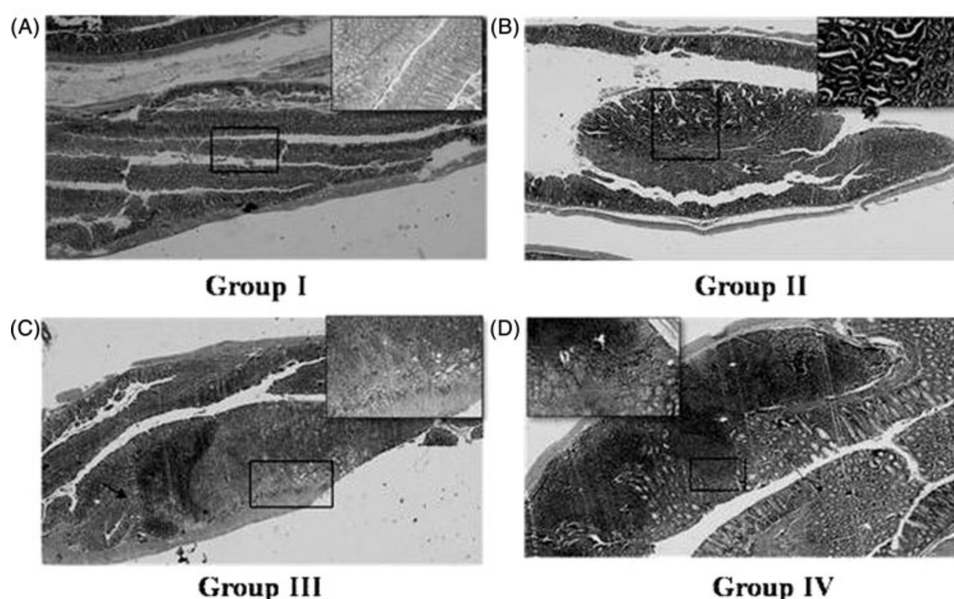

Figure 3. Histopathological sections of various groups. (A) Naïve control group, (B) DMH (positive control) group, (C) free GE treatment group and (D) GE beads treatment group.

Table 5. Micromeritic parameters of uncoated and coated GE beads[a].

Beads	Particle size (μm)	Angle of repose	Bulk density (g/cc)	Tap density (g/cc)	Hauser ratio
Uncoated	746.96 ± 17.34	32.01 ± 1.45	1.94 ± 0.08	2.17 ± 0.03	1.12 ± 0.03
Coated	895.36 ± 6.43	27.56 ± 2.08	2.11 ± 0.10	2.64 ± 0.05	1.25 ± 0.05

[a]All values for coated and uncoated beads are significantly ($p < 0.05$) different.

parameters in the pathogenesis of DMH-induced colon cancer as shown by significant ($p < 0.05$) increase in LPO (from 7.83 ± 0.38 moles of MDA/mg protein in control to 13.43 ± 1.08 moles of MDA/mg protein in DMH group), nitrite (5.94 ± 1.26 nmoles/mg protein in control to 31.41 ± 2.01 nmoles/mg protein in DMH group), MnSOD (1.93 ± 0.46 SOD units/mg protein in control to 3.34 ± 0.59 SOD units/mg protein in DMH group) and significant ($p < 0.05$) decrease in catalase (27.80 ± 3.89 μmol of H_2O_2 decomposed/mg protein in control to 9.00 ± 1.80 μmol of H_2O_2 decomposed/mg protein in DMH group) and CuZnSOD (7.36 ± 0.95 SOD units/mg protein in control to 3.82 ± 0.39 SOD units/mg protein in DMH group). Similar findings are reported earlier [26]. A significant reduction ($p < 0.05$) in the LPO (7.43 ± 0.32 moles of MDA/mg protein), nitrite (16.31 ± 3.81 nmoles/mg protein), and MnSOD (2.38 ± 0.47 SOD units/mg protein) levels and significant ($p < 0.05$) increase in catalase (13.91 ± 2.14 μmoles of H_2O_2 decomposed/mg protein) and CuZnSOD (4.82 ± 0.28 SOD units/mg protein) was observed for coated GE beads treated group

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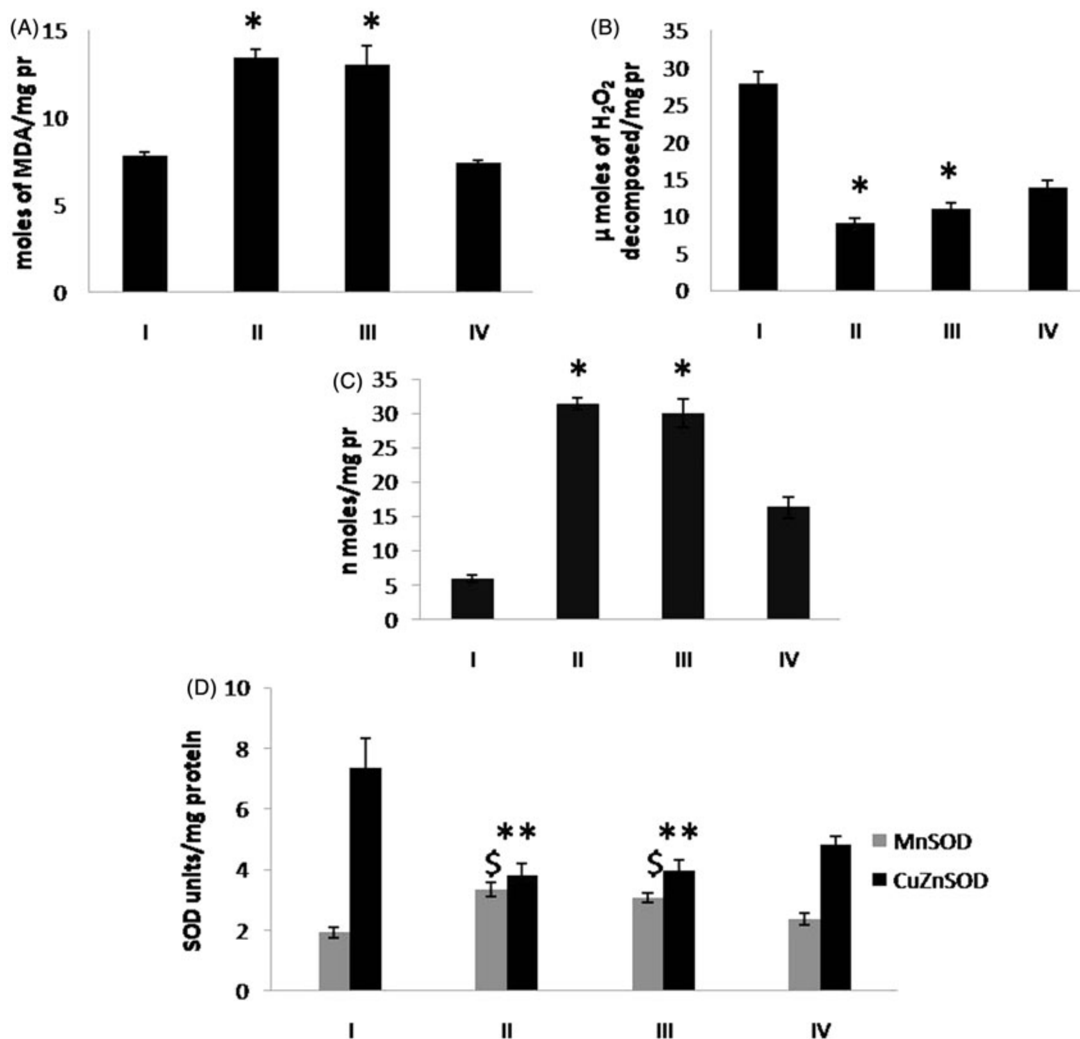

Figure 4. Effect of treatment on (A) LPO, (B) catalase, (C) nitrite and (D) SOD levels. All values are significantly ($p<0.05$) different from one another except for those marked similarly pr – protein.

when compared with the positive control group. The results obtained also indicate that the free GE did not produce any significant change in any of the biochemical marker with respect to the positive control group.

Mitochondrial enzyme complex estimation

Decrease in mitochondrial enzyme activity is another hallmark of colon cancer [27,28]. In line with these findings, significant ($p<0.05$) impairment in mitochondrial enzyme complex I (NADH dehydrogenase), complex II (succinate dehydrogenase) and complex IV (cytochrome oxidase) were observed. DMH treatment produced 64.81%, 53.09% and 25.14% decrease in complex I, II and IV activity, respectively (Figure 5).

About 15% of the complex I activity was restored with free GE, which was further increased to 23% when GE beads were administered. This increase may be attributed to the increased availability of GE at site of action, i.e. colon. Similarly, in case of complex II, enzyme activity improved by 17% and 27%, respectively, in groups III and IV as compared to the positive control group (group II). Neither free GE nor GE beads treatment could significantly restore any activity in the complex IV.

Ammonia and β glucuronidase determination

DMH treatment doubled ($p<0.05$) the ammonia levels in the colonic homogenates in comparison to the naive control (Figure 6A). Free GE did not produce any significant ($p<0.05$) change in ammonia levels. However, GE beads reduced the ammonia levels by 21%.

DMH treatment (group II) produced 1.74 times ($p<0.05$) increased β-glucuronidase activity in colonic homogenates as compared to naive control (group I) (Figure 6B). This increase remained unaltered when free GE was given for 1 month. However, β-glucuronidase activity was decreased by 14.45% in GE beads treated group (group IV).

Discussion

We tried to load two different concentrations of GE, i.e. 1% and 3% w/v into the alginate beads and we found that formulation G1 (with 3% w/v GE) showed better entrapment efficiency (Table 2). Thus we proceeded with beads G1 for developing a colon targeted system.

It is well documented that clinical colon cancer and also DMH induced colorectal tumors in rat are majorly observed in distal part of colon [24]. Same is supplemented with the

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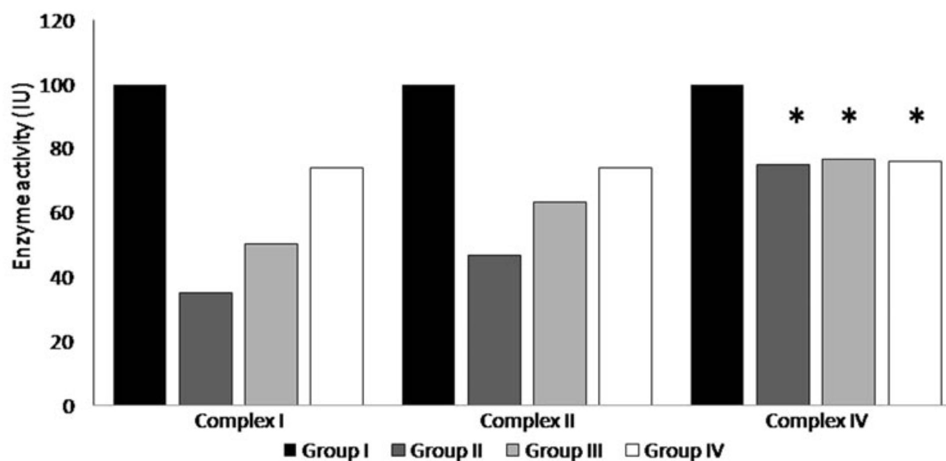

Figure 5. Effect of treatment on mitochondrial enzyme activity. Group I: naïve control; group II: DMH group; group III: free GE treatment group; group IV: GE beads treatment group. All values are significantly ($p < 0.05$) different from one another except for those marked similarly.

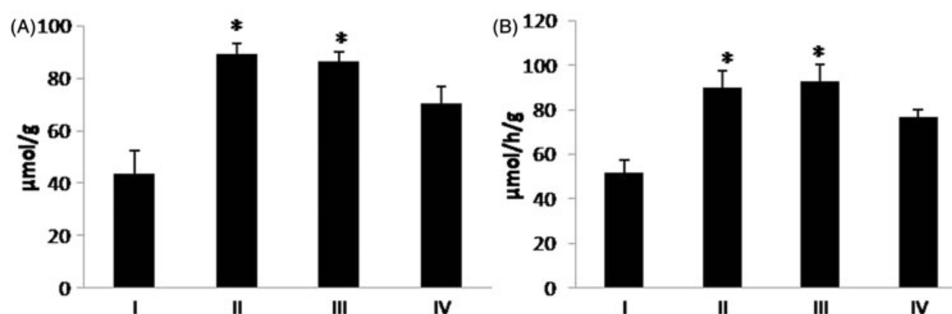

Figure 6. (A) Effect of treatment on ammonia concentration. (B) Effect of treatment on β glucuronidase activity. Group I: naïve control; group II: DMH group; group III: free GE treatment group; group IV: GE beads treatment group. All values are significantly ($p < 0.05$) different from one another except for those marked similarly.

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fact that ACF density is significantly higher in distal part than proximal colon [29]. Furthermore, the colon possesses unique characteristic of exhibiting a slightly acidic environment in the proximal part (pH 6.0–6.4) resulting from degradation of poly- and oligosaccharides to short chain fatty acids and slightly alkaline pH (pH 7.0–7.4) in its distal part [1,30]. Presently, we used Eudragit S100 as the coating polymer which dissolves at pH 7.0, thus targeting distal part of the colon.

On comparing *in vitro* release data for the coated (15% weight gain) and uncoated beads, it is evident that nearly half of the entrapped GE (47.26%) is released from coated beads after 12 h (vis a vis 21.25% from uncoated beads; $p < 0.05$) which means coating with Eudragit S100 ensures a majority GE delivery to the concerned target site, i.e. the distal part of colon.

It may, however, be noted that incorporation of GE into alginate beads is in itself restraining the release of GE and is common to both the types of beads such that they behave similarly in the stomach and upper GIT upto 5 h, when there is no statistically significant ($p < 0.05$) difference in their release pattern. Being a polyelectrolyte, alginate can exhibit swelling properties that are sensitive to the pH, ionic strength and ionic composition of the medium. Under acidic conditions swelling of calcium alginate beads occur scarcely [31]. The low swelling ratio in acidic pH (Table 4) is probably due to the proton-calcium ion exchange forming insoluble alginic acid regions due to solvent penetration into the gel network. Under neutral conditions the beads are expected to swell and the release largely depends on the swelling process. Increase in swelling at pH 6.8 is due to the increased solubility of the polymeric network. The swelling behaviour of GE-loaded Ca-alginate beads at higher pH may be explained by the ionotropy that occurs between Ca^{2+} ion of alginate and the Na^{+} ions present in phosphate buffer and the consequent capturing of the Ca^{2+} by the phosphate ions [32]. In other words, it may be said that swelling is enhanced by the presence of phosphate ions at higher pH which displace the Ca^{2+} ions within the beads [33].

The ion exchange with phosphate buffer which results in swelling and erosion of the beads and formation of the soluble Ca-phosphate influences the drug release rate at higher pH. In case of coated GE beads the ginger is released from alginate beads through swelling and erosion mechanism only after the eudragit coating dissolved.

Coated and uncoated GE beads were evaluated for various micromeritic parameters like particle size, angle of repose, bulk density, tap density and Hauser ratio (Table 5). Particle size of coated beads was significantly higher than uncoated beads. Angle of repose of coated beads was smaller than that of uncoated core beads, because of the rough surface of uncoated beads. Coating of beads also improved the packability, as depicted by increase in bulk and tap densities. The significantly improved micromeritic properties suggest that the developed beads can be easily handled.

Oxidative stress has been defined as a disturbance in the equilibrium status of pro-oxidant and antioxidant systems in

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favour of pro-oxidation. Oxidative stress develops particularly in inflammatory reactions because the inflammatory cells, neutrophils and macrophages produce large amounts of ROS. It has been known for a long time that oxidative stress in inflamed tissue can pave the way for malignant tumors, and that it is a major pathogenetic factor for the well-established correlation between inflammatory diseases and cancer [26].

Possible reasons assigned to colon cancer induced damage are as follows: (i) lipid peroxidation, oxidation of some critical cellular proteins, and depletion of antioxidants indicating production of ROS; (ii) activation of SOD which in turn favours endogenous accumulation of hydrogen peroxide; (iii) generation of oxygen ion at an enhanced rate during stress, as evidenced by increased SOD activity; and (iv) generation of hydroxyl ion at a higher rate from oxygen ion and hydrogen peroxide.

It has been reported that malonaldehyde (MDA) (determined in terms of thiobarbituric acid reactive substances (TBRAS)) acts as a mutagen and tumor promoter [34–36]. In the present study, DMH-treated rats showed a remarkable increase in the level of MDA and complete attenuation of induced LPO, expressed as MDA levels, was observed with coated GE beads treatment as the values match the naive control group ($p < 0.05$). This suggests an effective role of GE in checking lipid peroxidation, only when suitably delivered at the site.

SOD and catalase (CAT) are two important enzymatic antioxidants that act against toxic oxygen free radicals such as superoxide (O^{2-}) and hydroxyl (OH^-) ions in the biological system. They are involved in the direct elimination of reactive oxygen metabolites, which is probably one of the most effective defenses of the living body against diseases. SOD and CAT are reported to be more sensitive to oxidative damage induced by carcinogen treatment [37,38].

In eukaryotic cells, two forms of SOD are present; one contains both Cu and Zn (CuZnSOD) and the other contains Mn (MnSOD). The CuZnSOD is found mainly in cytosol and the MnSOD resides in the matrix of mitochondria. About 1–2% of inhaled O_2 may be converted to superoxide anion in mitochondria. MnSOD is an important enzyme in the protection of mitochondria from oxidative stress, since they do not have catalase activity [39]. MnSOD levels were increased significantly (1.73 times) in DMH-induced cancer group. It has been reported that the MnSOD isoform of SOD is inducible, whereas the synthesis of CuZnSOD is constitutive [40]. MnSOD messenger RNA (mRNA) is upregulated in intestinal epithelial cells by cytokines such as tumor necrosis factor (TNF) and interleukin 1 (IL-1) [41,42]. Increased TNF-α and IL-1 expression has been observed in rat colon cancer as compared with normal mucosa, supporting the hypothesis of autocrine induction of MnSOD [43].

Decreased activity of CuZnSOD and CAT in DMH treated rats suggests their increased utilization to scavenge the induced increase of ROS in the cancerous tissue. Ginger has been reported to spare these antioxidant enzymes due to its ability to scavenge free radicals and toxic carcinogenic electrophiles [44,45]. Enhancement in SOD and CAT levels in the colon may thus be explained by these facts.

Nitrite levels were significantly increased in DMH induced colon cancer group (group II) as compared to naive control (group I). Free GE treatment (group III) did not show any significant reduction in nitric oxide levels; whereas GE beads treatment (group IV) reduced the nitrite level by 52% as compared to the positive control group. This is again due to the increased availability of GE at the site of action by its entrapment within calcium alginate beads. Similar effects of ginger are reported earlier [46].

Decrease in mitochondrial enzyme activity is another hallmark of colon cancer [27,28]. Former studies have indicated some mutations in the mtDNA in different human cancers [47–49]. One of the major cellular generators of ROS is the electron transport chain of mitochondria, which include the hydroxyl free radical (OH^-), hydrogen peroxide, and superoxide anion. Mutations in mtDNA has been suggested to have the ability for actuating an increase in ROS levels, followed by deterioration of oxidative phosphorylation and mitochondrial respiration which leads to oxidative stress that can modify the DNA.

mtDNA code for various subunits of the mitochondrial enzyme complexes. Higher amounts of superoxide production *in vivo* have also been demonstrated in the cells with complex I deficiency [50,51]. Succinate dehydrogenase, or mitochondrial complex II, consists of four subunits, i.e. SdhA, SdhB, SdhC and SdhD and germline mutations in each of the components of complex II have been shown to disrupt complex formation and subsequently decrease the enzymatic activity of the remaining complex [52].

Complex IV (ferrocytochrome c, oxygen oxidoreductase) has three mitochondrial genomic encoded subunits [28]. Oxidative stress may produce mutations in any or all the subunits. Absence of any effect on complex IV activity on treatment with either free GE or GE beads may be due to the inability of GE to reverse or counter these mutations.

Intestinal microflora is involved in the production of carcinogens from procarcinogenic substances by bacterial enzymes. These include β-glucuronidase, β-glucosidase, azoreductase, nitroreductase, 7 α-steroid dehydrogenase and 7 α-hydroxy steroid dehydrogenase [53]. β-Glucuronidase is an enzyme responsible for the hydrolysis of glucuronides in the lumen of the gut. This reaction generates toxic and carcinogenic substances by hydrolysis of glucuronidase in the lumen of the gut. In this way, toxic aglycones can be regenerated *in situ* in the bowel by bacterial β-glucuronidase [54,55]. Our results suggest that GE may act as a prebiotic for the colonic beneficial bacteria resulting in the decreased activity of β-glucuronidase which was increased due to DMH treatment. The prebiotic effects of GE were experimentally confirmed by us using *Lactobacillus acidophilus* strain. A specific concentration (0.4% w/v) of GE when added to the culture media was found to improve the growth of *L. acidophilus* by a factor of log 2, in 96 h. Ammonia is a known tumor promoter [56] so suppression of ammonia formation in the gut as observed in the present study is also considered beneficial [57].

Conclusion

Presently, we prepared calcium alginate beads of GE by orifice ionic gelation method. The percentage drug entrapment and drug content of the formulated beads were found to

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be satisfactory by this method. In order to target colon, beads were coated with Eudragit S100 so that the release of GE in upper GIT could be retarded. The intact beads reached the ileocecal junction at 8 h. Release pattern from beads was found to be super class II controlled by swelling and relaxation of polymer. *In vivo* studies confirmed suitability and effectiveness of GE beads for the treatment of colon cancer post-induction. The study reaffirms our previous claim [7] that there is a need for the amalgamation in the knowledge banks of the life scientist, biochemist, and pharmacologist with the pharmaceutical formulation scientists to not only revalidate observational data of folklore with scientific rationale and mechanistic studies but also to try, improve and explore the full potential of these natural agents documented in the historic pool of east Asian countries like India, China and Japan. The experience of using these agents for several centuries not only validates their therapeutic potential but also establishes their safety. However, after giving them a pharmaceutical couture as claimed in the present study, it will be appropriate to recheck their safety. The formulation scientist should ensure improved stability, bioavailability including absorption and reduced clearance of such molecules. This can really enhance the therapeutic armamentarium available to the physicians for handling chronic, multifaceted, multitargeted diseases like cancer. Developed system can provide a suitable platform for spatial delivery of other herbal antioxidants also.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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