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Research Paper

Synbiotic (probiotic and ginger extract) loaded floating beads: a novel therapeutic option in an experimental paradigm of gastric ulcer

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Keywords
floating beads; ginger extract; histopathology; *Lactobacillus acidophilus*; stomach ulcers

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Abstract

Objective This study investigated the use of a bioactive phytochemical, namely ginger extract (GE), for its antioxidant and antiulcer effects, and also for supporting probiotic growth and activity. Use of probiotics is limited in therapy because of their transience and inability to survive the adverse physiological conditions of the gastrointestinal tract. Packaging probiotics in a suitably designed pharmaceutical system with GE may facilitate their establishment in the stomach mucosa.

Methods A probiotic (*Lactobacillus acidophilus*) and GE were simultaneously and individually encapsulated/immobilized in alginate floating beads. The developed system was evaluated for diameter, buoyancy, entrapment, porosity, in-vitro viability/release and pharmacodynamics in a cold restraint stress induced gastric ulcer model in rats.

Key finding The developed floating beads stayed in the stomach for more than 10 h and both agents were released slowly and over a prolonged period from these beads. Significant and promising results were obtained for the combination (synbiotic) system in terms of ulcer index, mucus secretion, oxidative stress and histopathological parameters, as compared with the individual agents. The developed system could completely revert the damage induced in ulcerated stomachs at physiological (ulcer index and mucus secretion), biochemical (oxidative stress) and histological levels.

Conclusion This study establishes that suitable packaging of GE and *Lactobacillus acidophilus* together in floating beads can help exploit their prospects as therapeutic curative agents rather than potential preventive agents.

Introduction

A synbiotic is a supplement that contains both a prebiotic and a probiotic, and the two work together to improve the 'friendly' flora of the human intestine. Oral administration of probiotics and prebiotics has proved beneficial in various gastrointestinal disorders. Probiotics, especially *Lactobacillus*, are useful in the treatment of gastric ulcers. They help to generate new epithelial cells, especially at the ulcerated margins, by decreasing the cell apoptosis to cell proliferation ratio.[1] However, the use of probiotics in therapy is limited because of adverse physiological conditions (e.g. acidic pH, mechanical stresses, digestive enzymes and bile acids) existing within the gut, which do not allow establishment of probiotics in the gut mucosa. These factors indicate a need to package probiotics into a suitable delivery system.

A multiple-unit floating drug delivery system is proposed as a promising delivery system for local gastric effects. However, the viability and metabolic activity of the entrapped probiotic needs to be maintained during any manipulation. We proposed the incorporation of ginger extract (GE), a non-conventional phytochemical prebiotic, into the system. In addition to enhancing the viability of the incorporated probiotic, GE also has antioxidant and anti-inflammatory effects,[2–4] and can therefore improve the efficacy and wider application of the combination product. The floating drug delivery system ensures the prolonged and continuous release of the probiotic in the stomach, allowing sufficient time for its adhesion and establishment on the gastric mucosa.[5] The idea of using natural agents with their own set of suitable therapeutic activity in addition to supporting probiotic growth in suitably designed pharmaceutical systems is relatively new. The use of GE as a prebiotic is not extensively studied or reported in the literature, and only one patent report exists in

this regard.[6] The present study reports on a new product with three novel benefits: a probiotic, a phytochemical prebiotic (GE) and a floating drug delivery system.

Materials and Methods

Materials

GE was a gift sample from Nisarga Biotech (Satara, India). The extract is claimed to be 100% natural; specific gravity 0.91 g/ml; refractive index 1.492; prepared under supercritical CO_2 extraction at 300 bar and 39°C. The gingerol and zingiberene content of the extract as determined by HPLC analysis was 16 and 6%, respectively. The procured GE was free from residual solvents. The probiotic, *Lactobacillus acidophilus* (LAB), was a gift sample from Ranbaxy (Gurgaon, India) (not less than 200 billion cfu/g). All other chemicals or reagents used in the study were of analytical reagent (AR) or guaranteed reagent (GR) grade.

Preparation of floating beads

Calcium alginate beads were prepared by an orifice-ionic gelation technique. A measured quantity of probiotic (20 mg; 2.66×10^9 cfu) or GE (3 g), or a combination of the two agents (probiotic + GE; synbiotic) was suspended/dissolved in water and PEG-400, respectively. The solution was dispersed in sodium alginate solution (3% w/v) containing hydroxypropyl methylcellulose (HPMC) (alginate/HPMC = 9 : 1 w/w). Calcium carbonate was added to the solution in a calcium carbonate/alginate ratio of 0.5 : 1.0 w/w. The mixture was degassed under bath sonication (20–30 min) to remove any entrapped air. The resulting solution was dropped through a 26-G syringe into 1% w/v calcium chloride solution containing 10% v/v acetic acid. The solution containing suspended beads was allowed to stir for 1 h to improve mechanical strength and to complete the reaction at room temperature. The formed beads were separated, washed with alcohol, then washed with distilled water and freeze-dried overnight using a freeze dryer maintained at −40°C. The product was lyophilized further for 6 h at −70°C.[7] Probiotic and synbiotic beads were prepared under aseptic conditions.

Characterization and evaluation of floating beads

Particle size

The particle size of the developed floating beads was determined using a particle size analyzer (Malvern Instruments Ltd, Malvern, UK).

Drug entrapment efficiency

To determine the drug entrapment efficiency of GE in GE and synbiotic floating beads, a measured amount of beads was crushed in a mortar and 15 ml methanol was added. The mixture was transferred to a tube, thoroughly vortexed and then centrifuged at 2000 rpm. Suitable dilutions were prepared in methanol and the samples were analysed spectrophotometrically at λ_{max} 281 nm against methanol as a blank, using a molecular extinction coefficient of 90 as per the previously validated method of analysis.[7] The % drug entrapment efficiency was calculated by the following equation:

$$\%\ \text{drug entrapment efficiency (GE)} = \text{actual GE content in beads}/\text{theoretical GE taken} \times 100$$

To determine the drug entrapment efficiency of LAB in the probiotic and synbiotic floating beads, a measured amount of beads was triturated in a sterile mortar using a small quantity of sterile peptone water, making the final volume to 10 ml. Serial dilutions of the latter were plated on MRS agar (pour plate) and incubated anaerobically at 37°C for 48 h. The number of colony forming units (cfu) was counted and entrapment/viability determined as:

$$\%\ \text{drug entrapment efficiency (LAB)} = \log(\text{cfu obtained from beads})/\log(\text{cfu that should be ideally obtained}) \times 100$$

Buoyancy and porosity

The buoyancy of the beads was determined using a USP type II dissolution test apparatus. Fifty beads were placed in the vessel containing 500 ml simulated gastric fluid and maintained at 37 ± 0.5°C and stirred at 100 rev/min[8] for 24 h. The number of beads settling down after 24 h was measured by visual observation and the percentage of beads that remained floating was determined.

The porosity of the beads was determined as reported previously.[7] Briefly, beads were added to a 10-ml graduated measuring cylinder up to the 10-ml mark (taken as bulk volume) and the cylinder was tapped 500 times. The volume thus obtained indicated the tap volume of the beads. The porosity of the developed beads was calculated as:

$$\text{porosity} = V_b - V/V_b \times 100$$

where V_b is the bulk volume of the beads (10 ml); V_p is the true/tap volume of the beads; and V is the void volume of the particles (spaces between particles; $V = V_b - V_p$).

Surface characterization

The external and internal morphology of the freeze-dried floating beads was studied by scanning electron microscopy. Samples were coated with gold film under vacuum before investigation. The internal morphology of the beads was examined by cutting them in half with a steel blade.

In-vitro release study for GE floating beads

The drug release study was carried out in a USP type II dissolution test apparatus, with 900 ml simulated gastric fluid (pH

1.2), at 100 rev/min and 37 ± 0.2°C using floating beads equivalent to 84.55 mg of GE. Suitable fractions (5 ml) were withdrawn at various time intervals and replaced with fresh medium. Samples were analysed spectrophotometrically for GE content.

In-vitro release and viability studies for probiotic floating beads

The release and viability study of the probiotic from prepared beads was carried out in simulated gastric fluid under aseptic conditions. Several tubes containing 100 mg of beads/free probiotic suspended in 10 ml simulated gastric fluid were incubated anaerobically at 37°C. All the beads were removed from respective tubes at regular intervals, washed with peptone water and immediately assayed for cell count to give a measure of viability. Supernatant from each tube was also evaluated for cell count and gave a measure of LAB released from the beads with time. Tubes containing free probiotic were centrifuged and the cell count in pellets and supernatants were used similarly. All the experiments were carried out under aseptic conditions.

Cold restraint stress induced gastric ulcers

Animals

Female Wistar rats, not more than 250 g, bred in the Central Animal House, Panjab University (Chandigarh, India) were used. Animals were deprived of food but were allowed free access to water 24 h before the start of the experiment. The experimental protocol was approved by the Institutional Animal Ethics Committee, Panjab University (reference no. 972/DUI/PU, 2010).

Experimental procedure

Animals were divided into 21 groups; each group consisting of five animals (Table 1). Group 1 comprised naive control animals (24-h fasted animals). All other animals were immobilized (by strapping the fore and hind limbs on a wooden plank) and kept for 3 h at a 4 ± 1°C,[9] to generate cold restraint stress (CRS), which is reported to induce gastric ulcers mainly through the generation of oxidative stress.[10] Immediately after release, all animals except the control groups (2, 10 and 14), were orally administered with the appropriate treatment (Table 1). Animals were killed at intervals of 2 h (Groups 1–9), 4 h (Groups 10–13) and 10 h (Groups 14–21) by cervical dislocation.

Determination of ulcer index and haemorrhagic streaks

The ulcer index was calculated by adding the total number of ulcers plus the severity of ulcers judged based on a scale.[11]

Table 1 Treatment schedule of different groups in the cold restraint stress study

Time	Group no.	Treatment
2 h	1	Naive control
	2	CRS
	3	CRS + free GE (200 mg/kg)
	4	CRS + free probiotic (10^7 cfu)
	5	CRS + free GE (200 mg/kg) and probiotic (10^7 cfu)
	6	CRS + cimetidine (10 mg/kg)
	7	CRS + GE floating beads (equivalent to 200 mg/kg)
	8	CRS + probiotic floating beads (equivalent to 10^7 cfu)
	9	CRS + synbiotic floating beads (equivalent to 200 mg/kg GE and 10^6 cfu)
4 h	10	CRS
	11	CRS + GE floating beads (equivalent to 200 mg/kg)
	12	CRS + probiotic floating beads (equivalent to 10^7 cfu)
	13	CRS + synbiotic floating beads (equivalent to 200 mg/kg GE and 10^6 cfu)
10 h	14	CRS
	15	CRS + free GE (200 mg/kg)
	16	CRS + free probiotic (10^7 cfu)
	17	CRS + free GE (200 mg/kg) and probiotic (10^7 cfu)
	18	CRS + cimetidine (10 mg/kg)
	19	CRS + GE floating beads (equivalent to 200 mg/kg)
	20	CRS + probiotic floating beads (equivalent to 10^7 cfu)
	21	CRS + synbiotic floating beads (equivalent to 200 mg/kg GE and 10^6 cfu)

Rats were killed at 2, 4 and 10 h. CRS, cold restraint stress; probiotic, *Lactobacillus acidophilus*; GE, ginger extract.

The sum of the respective lengths of various haemorrhagic streaks was also measured and used as another parameter for assessing the extent of ulcers.

Histopathological examination

For histopathological examination of gastric mucosal lesions, stomachs were opened along the greater curvature, fixed in a 10% buffered formalin solution, embedded in paraffin and microtomed. Sections of 5-μm thickness were cut and stained with hematoxylin and eosin. The specimens were observed under a high-power light microscope and evaluated for mucosal lesions.

Estimation of gastric wall mucus

The glandular segments of respective stomachs were scraped with a blunt spatula, weighed and incubated in tubes containing 0.1% alcian blue solution (0.16 M sucrose in 0.05 M sodium acetate, pH 5.8) for 2 h.[12] The alcian blue binding extract was centrifuged and the absorbance of supernatant was measured at 598 nm. The quantity of alcian blue extracted (μg/g glandular tissue) was then calculated.

Biochemical analysis of stomach homogenates

Removed stomachs were rinsed with ice-cold saline and weighed. A 10% w/v stomach homogenate was prepared in 0.1 M phosphate buffer saline (pH 7.4) and was used for determination of lipid peroxidation (LPO),[13] catalase (CAT),[14] superoxide dismutase (SOD)[15] and protein estimation.[16]

Statistical analysis

The raw data obtained from in-vitro studies is expressed as mean ± SD. The in-vivo results are expressed as mean ± SEM. The intergroup variation was measured by one-way analysis of variance followed by Tukey's test. All statistical tests were performed for the control values (2, 4 and 10 h) and the values obtained for the 10-h groups.

Results

Characterization and evaluation of floating beads

The average particle size of the GE, probiotic or synbiotic floating beads was around 1 mm in all cases (Table 2). The buoyancy of the prepared beads was found to vary from 77% to 80% (Table 2). The drug entrapment efficiency of GE and probiotic loaded floating beads was 84.5% and 84.7%, respectively. In the case of synbiotic floating beads, the drug entrapment efficiency of GE was 92.8%, however entrapment of the probiotic was 87.3% as shown in Table 2. The porosity of GE, probiotic and synbiotic floating beads was between 76% and 92% as shown in Table 2. The surface and cross-sectional pictures of GE and probiotic loaded floating beads and synbiotic floating beads are shown in Figure 1. Floating beads had a wrinkled surface due to the release of carbon dioxide from the surface of beads during their formation. The cross-sectional views of the beads revealed several closed channels or pores (Figure 1).

In-vitro dissolution study

Free GE showed slower and lesser release in comparison with GE floating beads. GE showed poor water solubility (0.69 mg/ml) which was increased by almost 4-times to 2.63 mg/ml in the components used to prepare floating beads.[7] Thus, it may be concluded that GE is restrained within the floating beads in a soluble form and the release studies indicated a slow and Fickian release (i.e. diffusion controlled release from the developed beads) (Figure 2a).

Release and viability of probiotic from floating beads

Figure 2b and 2c show the variation of bacterial counts (in terms of viability and % released from the beads) after exposure of free probiotic or probiotic beads to simulated gastric fluid at 37°C for 6 h. The % viable number of free probiotic and probiotic entrapped within floating beads decreased significantly with time. The rate constant of decline (K) in the bacterial count at all time points for probiotic floating beads was significantly less ($P < 0.05$) than free probiotic (Table 3), with the value being almost 1.7- and 1.5-times less at 2 and 4 h, respectively. This confirmed that encapsulation of these probiotic bacteria within floating beads protected them from the harsh acidic conditions in gastric fluids. The release data also indicate the advantage of the developed delivery system. The value of K was given by the following equation:

$$K = \log(a/(a-x)t)$$

where K represents the rate constant of decline, a is the initial number of bacteria in the medium, and $(a - x)$ is the number of bacteria in the same volume after exposure for time t.

Cold restraint stress induced gastric ulcers

Studies were performed at 2, 4 and 10 h. The first two time points represent the normal gastric transit time of 2–4 h for free drugs (GE, probiotic, GE + probiotic), while the 10-h time point represents the prolonged stay achieved with floating beads. Opening the stomach at different time points post-administration revealed significant retention of beads (22%) within the stomach, even at 10 h as shown in Figure 3.

Ulcer index and haemorrhagic streak length

A significant ulcer index of >17 was observed in the CRS groups (Groups 2, 10 and 14) (Table 4). Treatment of CRS rats with cimetidine (H2 receptor antagonist used in the study for comparison), GE, probiotic, GE together with probiotic, and the respective floating bead formulations (GE, probiotic and GE + probiotic) significantly reduced the

Table 2 Results of in-vitro studies on batches of differently loaded floating beads ($n = 3$)

Batch	Particle size (mm)	Drug entrapment efficiency (%)	Buoyancy (%)	Porosity
B1	1.09 ± 0.03	84.55 ± 3.43	77.33 ± 2.31	86.67 ± 1.53
B2	1.04 ± 0.02	84.71 ± 2.52	80.00 ± 1.15	76.33 ± 1.45
B3	1.09 ± 0.18	92.83 ± 1.49 (ginger extract) and 87.29 ± 3.37 (probiotic)	77.33 ± 1.76	91.67 ± 1.45

B1, ginger extract loaded floating beads; B2, probiotic loaded floating beads; B3, synbiotic (probiotic + ginger extract) loaded floating beads.

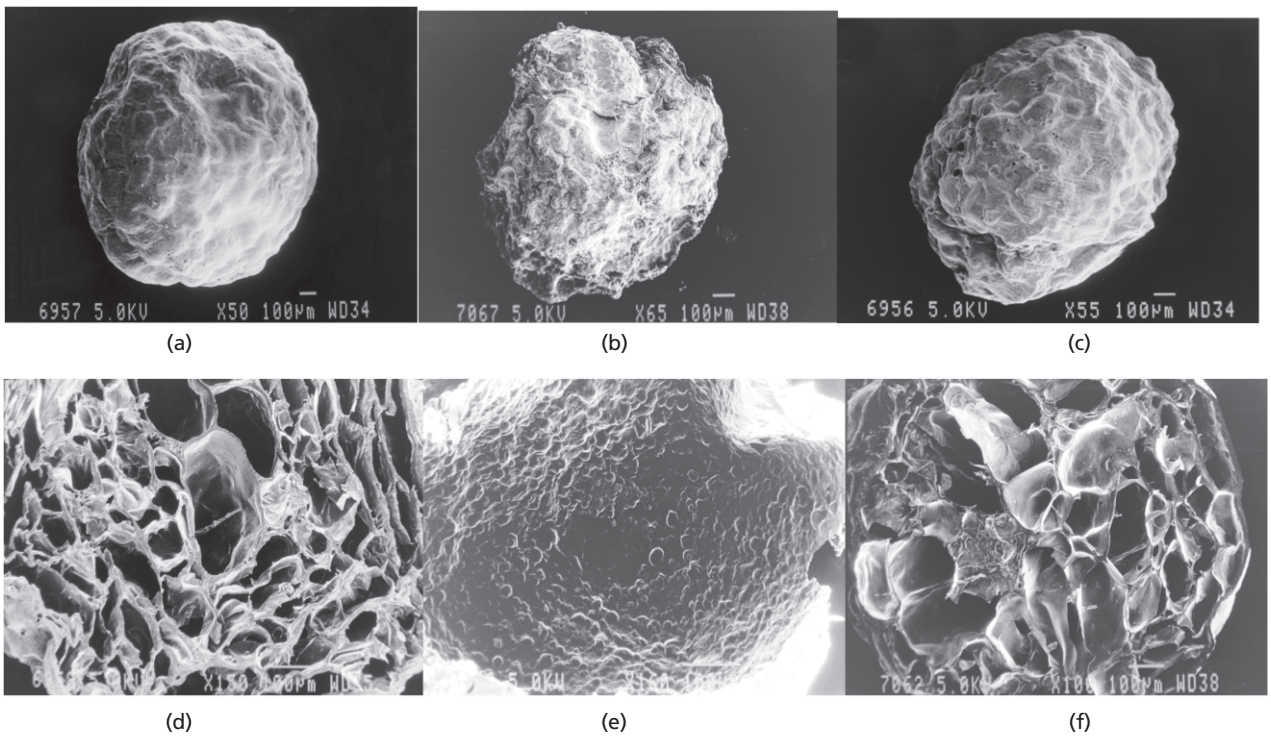

Figure 1 Scanning electron microscope photographs of different formulations. (a) Ginger extract loaded floating beads; (b) probiotic loaded floating beads; (c) synbiotic floating beads; (d) cross-section of ginger extract loaded floating beads; (e) cross-section of probiotic loaded floating beads; (f) cross-section of synbiotic floating beads.

number as well as the extent of gastric mucosal ulcers. Synbiotic loaded floating beads were significantly ($P < 0.05$) more effective in reducing haemorrhagic streak length as compared with cimetidine, GE floating beads and probiotic loaded floating beads. In terms of ulcer index, synbiotic loaded floating beads were significantly ($P < 0.05$) more effective as compared with cimetidine, but no significant difference was observed between synbiotic, GE and probiotic loaded floating beads (2.25 ± 0.72 for synbiotic floating beads; 3.63 ± 0.63 for probiotic floating beads; 4.00 ± 0.46 for GE floating beads). The results establish the therapeutic usefulness of all these agents upon incorporation in floating beads, given that they could alleviate the ulcerative damage induced by CRS.

Histopathological examination

Histopathological study of gastric mucosa was performed after staining with haematoxylin and eosin. Figure 4a shows the normal healthy mucosa of a naive control animal, with the mucosal glands maintaining their identity. The CRS induced group showed mucosal hyperaemia and haemorrhagic necrotic lesions, with oedema covering the entire glandular area of the stomach (as outlined on the figure), indicating acute ulceration as shown in Figure 4b. In addition, gastric mucosal damage with dilation and exfoliation of gastric epithelial cells and disruption of the mucosal layer was observed, and the glands were also found to lose their identity. Post-treatment with free GE (200 mg/kg) at the end of 10 h reduced the lesions as compared with the untreated group, but more than half of the mucosal surface was still damaged as shown in Figure 4c. The cimetidine (10 mg/kg) treated group at the end of the 10-h study showed better recovery of gastric mucosa as compared with the free GE treated group, with most parts of the mucosa regaining its identity except the edges as shown in Figure 4d. After treatment with free probiotic, the mucosa showed slight recovery but significant mucosal damage was still obvious, coupled with inflammation inside the mucosal cells as shown in Figure 4e. The GE floating beads (equivalent to 200 mg/kg) treated group showed better recovery than cimetidine and free GE treated groups, with the gastric mucosa regaining its identity and starting to maintain its architecture as shown in Figure 4f. Reddish bloody portions associated with a high degree of inflammation were still apparent after treatment with probiotic floating beads, however the extent of inflammation was less with respect to the free probiotic, confirming the anti-ulcerative effect of probiotic floating beads as shown in Figure 4g. The histopathological examination in the case of

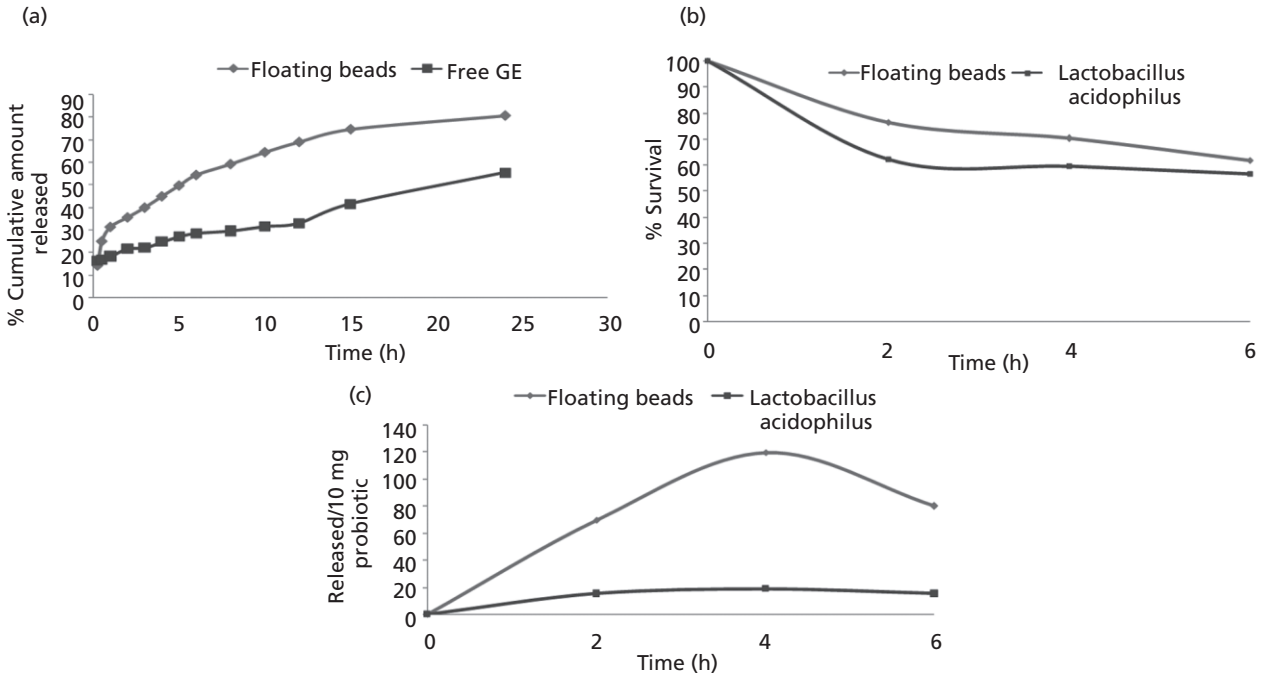

Figure 2 (a) Comparative in-vitro release profile of free ginger extract (GE) and GE loaded floating beads. (b) Comparative % survival of *Lactobacillus acidophilus* and *L. acidophilus* loaded floating beads. (c) Comparative in-vitro release profile of *L. acidophilus* and *L. acidophilus* loaded floating beads in simulated gastric fluid.

Table 3 Rate constant of decline of free probiotic and probiotic loaded floating beads in simulated gastric fluid

	K value ($n = 4$)	
Time (h)	Free probiotic	Probiotic floating beads
2	–0.1135	–0.2147
4	0.1072	–0.0857
6	0.3766	0.0033

Values for the free probiotic group were significantly different compared with the probiotic floating beads group at each time point ($P < 0.05$).

the free synbiotic treated group showed significant damage of the inner mucosa, with the latter losing its identity. A high degree of inflammation was also apparent. The degree of inflammation was greater than that observed for the group treated with probiotic beads as shown in Figure 4h. The group treated with the synbiotic floating beads showed almost complete recovery from ulcers. No inflammation or mucosal damage was visible either at the edges or in the inner parts of mucosal cells, thus confirming the anti-ulcerative effect of developed floating beads as shown in Figure 4i.

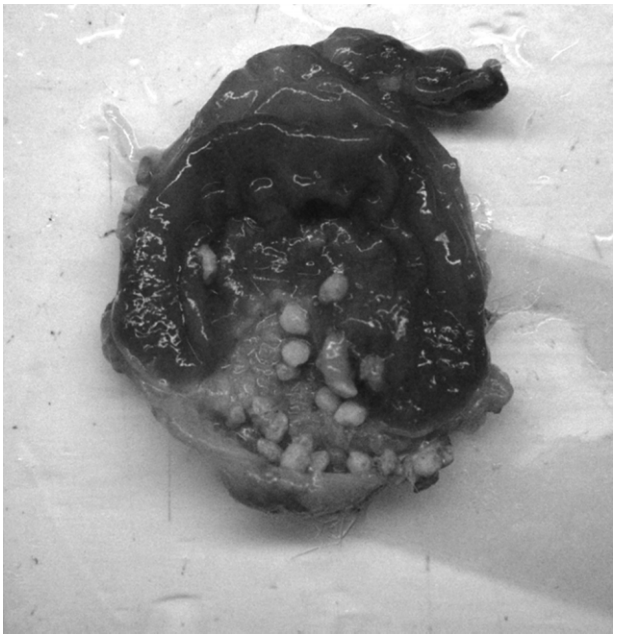

Figure 3 Rat stomach containing floating beads at the end of the 10-h study.

Mucus content determination

Treatment with the free drugs (GE, probiotic, GE + probiotic, cimetidine) significantly ($P < 0.05$) suppressed the decrease

Table 4 Effect of various treatments on ulcer index, haemorrhagic streak length, mucus content, lipid peroxidation, superoxide dismutase and catalase levels

Time	Group	Ulcer index	Haemorrhagic streak length (mm)	Mucus content (μg/g tissue)	Lipid peroxidation (nmol/mg protein)	Superoxide dismutase (units/mg protein)	Catalase (μmol H_2O_2 decomposed/min per mg protein)
2 h	1	0	0	287.8 ± 8.7	3.8 ± 0.6	11.0 ± 1.2	23.6 ± 0.9
	2	18.0 ± 1.0[a]	90.8 ± 5.0[a]	127.5 ± 8.4[a]	13.0 ± 0.8[a]	2.1 ± 0.5[a]	7.7 ± 1.9[a]
	3	13.0 ± 1.2	81 ± 1.7	143.9 ± 5.7	9.2 ± 1.0	3.7 ± 0.7	11.6 ± 0.8
	4	11.0 ± 1.7	75.3 ± 4.1	135.3 ± 10.1	10.6 ± 0.7	4.0 ± 1.1	12.1 ± 1.2
	5	10.3 ± 1.0	71.0 ± 4.0	140.5 ± 7.9	8.3 ± 0.4	2.5 ± 0.3	12.0 ± 0.6
	6	12.0 ± 1.5	73.3 ± 7.6	148.6 ± 7.5	8.1 ± 0.7	3.2 ± 0.4	12.4 ± 0.7
	7	13.0 ± 1.1	75.5 ± 5.6	154.5 ± 6.8	8.7 ± 0.8	3.2 ± 0.7	12.1 ± 0.7
	8	13.8 ± 0.9	78.8 ± 5.0	157.4 ± 11.8	9.0 ± 0.9	4.0 ± 1.4	12.5 ± 0.7
	9	10.0 ± 1.5	72.5 ± 3.2	161.7 ± 12.0	8.5 ± 0.2	3.9 ± 0.9	12.3 ± 0.6
4 h	10	17.8 ± 1.4[a]	90 ± 7.8[a]	130.9 ± 3.3[a]	13.6 ± 1.6[a]	2.3 ± 0.6[a]	9.7 ± 0.9[a]
	11	8.5 ± 1.0	58 ± 5.3	173.1 ± 9.3	7.1 ± 0.4	4.0 ± 0.6	15.0 ± 1.0
	12	8.0 ± 1.3	60 ± 6.6	187.1 ± 11.4	6.3 ± 0.4	3.4 ± 0.4	14 ± 1.1
	13	5.8 ± 0.6	42.5 ± 4.0	200.3 ± 10.9	5.5 ± 0.4	4.5 ± 0.6	16.1 ± 0.7
10 h	14	17.6 ± 1.2[a]	91.6 ± 6.0[a]	131.7 ± 6.3[a]	13.3 ± 1.5[a]	2.3 ± 0.6[a]	9.2 ± 0.9[a]
	15	11.3 ± 1.0[a,b]	71.3 ± 3.2[a]	159.9 ± 9.7[a]	7.7 ± 0.8[a,b]	3.5 ± 0.6[a]	13.1 ± 1.1[a]
	16	8.7 ± 1.5[a,b]	67.7 ± 8.4[a]	151.8 ± 7.8[a]	7.3 ± 0.6[a,b]	5.0 ± 1.2[a]	13.1 ± 1.5[a]
	17	7.3 ± 1.5[a,b]	56.7 ± 5.4[a,b]	156.9 ± 10.8[a]	6.3 ± 0.3[a,b]	4.1 ± 0.7[a]	14.7 ± 0.6[a]
	18	7.8 ± 1.3[a,b]	39.5 ± 5.1[a,b,c,d]	172.7 ± 4.5[a]	7.8 ± 0.6[a,b]	4.2 ± 0.7[a]	15.3 ± 0.9[a]
	19	4.0 ± 0.5[a,b,c]	33 ± 9.0[a,b,c,d]	205.7 ± 9.1[a,b,c]	6.3 ± 0.5[b]	7.7 ± 0.7[b]	19.8 ± 2.0[b,d]
	20	3.6 ± 0.6[a,b,c,d]	36 ± 3.8[a,b,c,d]	198.5 ± 8.1[a]	6.0 ± 0.8 [b]	7.0 ± 1.1	15.4 ± 1.1[a]
	21	2.3 ± 0.7[a,b,c,d,e]	14.5 ± 2.2[a,b,c,d,e]	235.6 ± 9.3[a,b,d]	4.4 ± 0.8[b,c,d]	9.1 ± 1.8[b,c]	20.1 ± 1.1[b,c,d]

Rats were killed at 2, 4 and 10 h. [a]$P < 0.05$ as compared with Group 1, [b]$P < 0.05$ as compared with positive controls, Groups 2, 10 and 14. Ulcer index: [c]$P < 0.05$ as compared with Group 15, [d]$P < 0.05$ as compared with Group 17, [e]$P < 0.05$ as compared with Group 18. Haemorrhagic streak length: [c]$P < 0.05$ as compared with Group 15, [d]$P < 0.05$ as compared with Group 16, [e]$P < 0.05$ as compared with Group 17. Mucus content: [c]$P < 0.05$ as compared with Group 16, [d]$P < 0.05$ as compared with Groups 15, 17, 18. Lipid peroxidation: [c]$P < 0.05$ as compared with Group 15, [d]$P < 0.05$ as compared with Group 18. Superoxide dismutase: [c]$P < 0.05$ as compared with Group 15, 17 and 18. Catalase: [c]$P < 0.05$ as compared with Group 15, [d]$P < 0.05$ as compared with Group 16.

in mucus levels. Probiotics are reported to stimulate mucus secretion and increase transmucosal resistance in gastric mucosa.[1] Loading of drugs into floating beads significantly improved their efficacy in comparison with the corresponding free drugs ($P < 0.05$; except probiotic floating beads). The synbiotic loaded floating beads were significantly more effective as compared with cimetidine, while the effect was similar to that observed for GE floating beads and probiotic loaded floating beads as shown in Table 4.

Oxidative stress

We determined LPO, CAT and SOD levels for the cimetidine, free drugs and the respective floating bead formulations (GE, probiotic, GE + probiotic) as markers of oxidative stress in CRS induced rat stomach homogenates and compared the values obtained with naive control values.

Lipid peroxidation

In order to verify the increase in LPO induced by CRS, thiobarbituric acid reacting substance (TBARS) levels were measured and were found to be significantly higher in the CRS treated group ($P < 0.05$) as compared with the naive control. The elevated TBARS levels were attenuated upon treatment with GE, probiotic, cimetidine, GE with probiotic, and also with the floating beads of the free drugs individually and combined (synbiotic floating beads). Among these, synbiotic loaded floating beads were significantly ($P < 0.05$) more effective as compared with free drug and cimetidine; LPO levels approached normal values after 10 h of administration as shown in Table 4.

Superoxide dismutase activity

Significantly decreased SOD activity was observed in the CRS treated group, which could be due to the utilization of SOD to combat the increased production of O_{2-} subsequent to cold stress. Administration of free drugs (GE, probiotic, GE with probiotic, and cimetidine) and their respective floating bead formulations (GE, probiotic and synbiotic floating beads) seemed to effectively scavenge reactive oxygen species generated by cold stress, thus sparing SOD. Results showed that synbiotic loaded floating beads were significantly ($P < 0.05$)

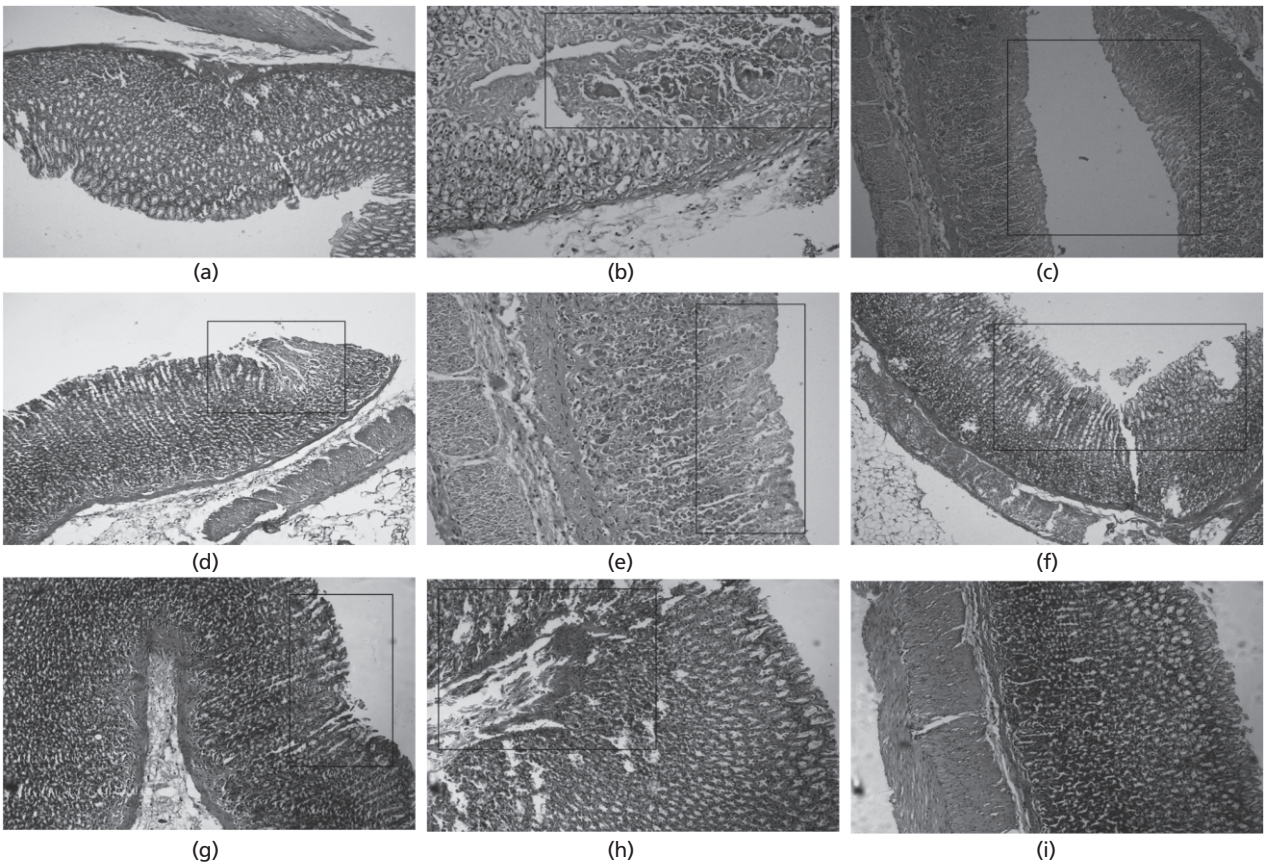

Figure 4 Histopathological micrographs of rat stomach from different treatment groups at the end of the 10-h study. (a) Naive control; (b) cold restraint stress; (c) ginger extract; (d) cimetidine; (e) probiotic; (f) ginger extract floating beads; (g) probiotic floating beads; (h) free ginger extract and probiotic; (i) synbiotic floating beads.

more effective than cimetidine, but no significant difference was observed between probiotic loaded floating beads and GE floating beads as shown in Table 4.

Catalase activity

A significant ($P < 0.05$) decrease in CAT activity in the CRS treated group as compared with the naive control was observed and was probably due to its extensive utilization in handling H_2O_2 overproduction in CRS. Administration of GE, cimetidine, probiotic, GE with probiotic and their respective floating bead formulations increased CAT levels. GE loaded floating beads showed significantly better effects than the free probiotic at $P < 0.05$. Further results showed that treatment with synbiotic floating beads was significantly ($P < 0.05$) more effective than treatment with free probiotic and free GE. Furthermore, the values obtained at 10 h were found to be similar to the naive control, indicating a complete reversal of induced damage as shown in Table 4.

Correlation of oxidative stress markers with ulcer index

Oxidative stress is believed to initiate and aggravate gastric mucosal damage and ulcers. In order to correlate the significance of reduced oxidative stress parameters as mentioned above, we plotted the ulcer index of various animal groups (Group 1; Groups 14–21) against these biochemical parameters. The correlation coefficients obtained from the plots indicated significant associations ($r^2 = 0.753$–0.907) with a maximum interrelation being observed for malondialdehyde (MDA) levels and ulcer index. A positive relationship was observed between ulcer index and MDA levels (LPO), while a negative relationship existed between the ulcer index and SOD, CAT and mucus content (Figure 5).

Discussion

The novel idea of using natural agents such as GE with their own therapeutic activity in addition to supporting probiotic

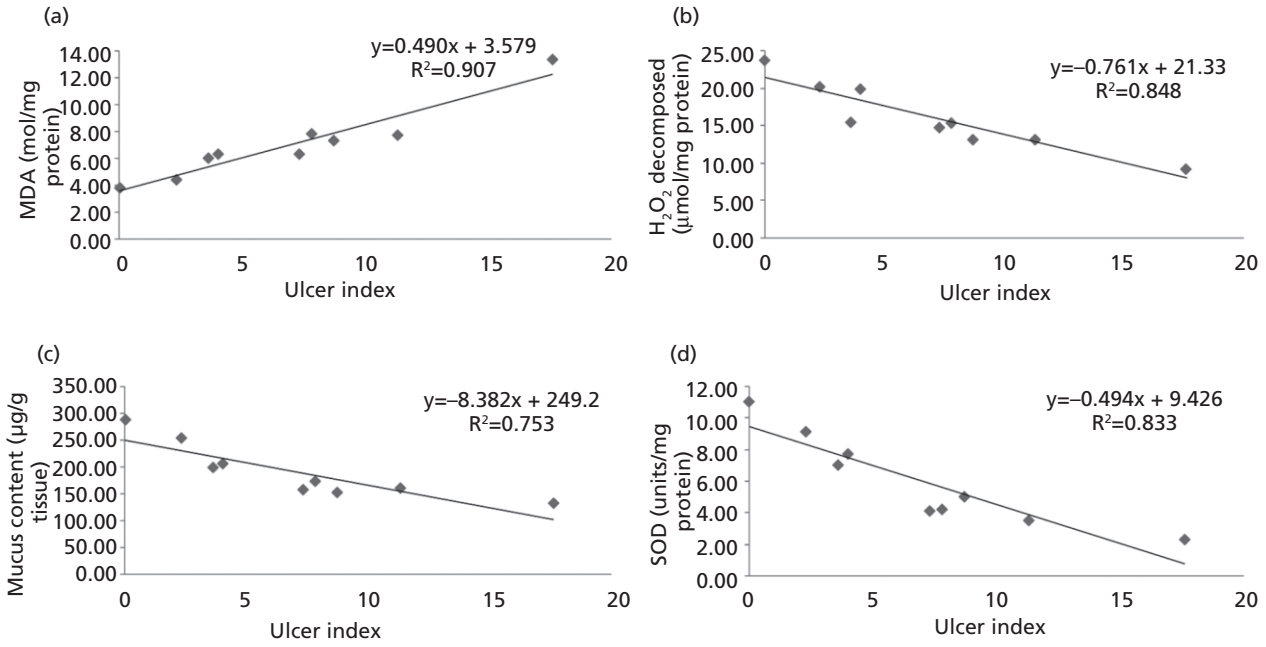

Figure 5 Correlation between ulcer index and oxidative stress markers and mucus content. (a) Ulcer index versus lipid peroxidation (MDA); (b) ulcer index versus catalase; (c) ulcer index versus mucus content; (d) ulcer index versus superoxide dismutase (SOD).

growth in suitably designed pharmaceutical systems was proposed. Most of these agents have shown promise in several in-vitro studies and also upon longterm administration *in vivo* before the induction of disease. The scientific community has as a result assumed these agents to be preventives based on experimental data complemented with dietary epidemiological studies. We strongly believe that these agents can be developed as therapeutics if focus is shifted towards suitably modulating the limiting physicochemical and pharmacokinetic nature of these agents. These limiting factors often reduce the desired efficacy of these agents *in vivo*. Given this and the limited survival and establishment of probiotics expected in the gastric mucosa, we developed GE, probiotic and synbiotic floating beads for post-induction protective effects against CRS induced gastric ulcers. During the formation of beads, calcium carbonate effervesces releasing carbon dioxide which is entrapped in the gel network (HPMC/alginate), producing a formulation that remains buoyant for prolonged periods. More than 75% of the beads were buoyant after 24 h, such that GE and LAB incorporated within them produce a pronounced and prolonged local effect as compared with the free drugs. The much higher porosity of synbiotic beads as compared with the individual drug loaded beads could be due to significant entrapment of both agents. The more frequent occurrence and larger pore size was also evident in the cross-sectional picture of the synbiotic beads. The beads (Figure 3) seemed to be adhering to the gastric mucosa, which may be due to the use of HPMC/sodium alginate in the preparation of floating beads as both of these agents are reported to be mucoadhesives.[17] The sustained release of probiotic from beads close to the gastric mucosa for prolonged times may facilitate their adherence to the gastric mucosa, giving them enough time and space to colonize such that significantly better effects are obtained.

The in-vitro dissolution study showed that the cumulative percentage release of GE from entrapped beads was more than 80% at the end of 24 h (Figure 2a). Further, the rate of death of LAB at all time points was significantly less than free LAB, confirming that encapsulation within floating beads protected them from the harsh acidic conditions, maintaining their viability in the gastric environment and improving their release characteristics from the developed floating beads.

CRS induces gastric mucosal damage possibly due to oxidative stress. The involvement of reactive oxygen species in gastric mucosal damage and ulcers is established. Oxidative damage induced increase in rat gastric mucosal LPO and SOD, and decrease in CAT levels, in CRS induced gastric ulceration is reported.[18] Cold stress leads to a significant decrease in mucus content[19] and an increase in prostaglandin levels[20] in rat stomach. Mucus is an important protective factor for the gastric mucosa. Moreover, mucus is capable of acting as an antioxidant and thus can reduce the mucosal damage mediated by oxygen free radicals. Mucosal damage increases gut permeability to macromolecules and facilitates the translocation of noxious materials such as carcinogens, endotoxins and other bacterial toxins to the bloodstream.[21]

The changes in LPO, CAT, SOD, mucus content levels, and ulcer index as well as haemorrhagic streaks induced by stress were restored to normal values by synbiotic floating beads. It may be noted that the cfu level of the probiotics in this system was 10^6, while the free probiotic, probiotic + GE system and probiotic floating beads system constituted of 10^7 cfu. Inspite of lower cfu of probiotic the synbiotic floating beads system showed a significantly better effect, indicating mutual synergism of GE with the probiotic, which was not so apparent when the two agents were administered together in the free from. Histopathological studies also indicated that synbiotic floating beads resulted in almost complete recovery from ulcers. No inflammation or mucosal damage was visible at the edges or the inner part of mucosal cells, confirming the anti-ulcerative effect of developed system.

Conclusions

The results suggest that synbiotic floating beads may provide a new, effective and powerful therapeutic strategy to treat gastric ulcers. To date, probiotics and natural antioxidants/phytochemicals have been mostly promoted for their protective effects. The synbiotic system developed in this study shows their potential to completely revert the damage induced in ulcerated stomachs at physiological (ulcer index and mucus secretion), biochemical (oxidative stress markers) and histological levels. The change in ulcer index correlated well with biochemical parameters and mucus content. Elucidating the effect of synbiotic floating beads in *Helicobacter pylori* induced ulcers may further establish the usefulness of these systems.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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References

1. Lam EK *et al.* Probiotic *Lactobacillus rhamnosus* GG enhances gastric ulcer healing in rats. *Eur J Pharmacol* 2007; 565: 171–179.
2. Goto C *et al.* Lethal efficacy of extract from *Zingiber officinale* (traditional Chinese medicine) or [6]-shogaol and [6]-gingerol in Anisakis larvae in vitro. *Parasitol Res* 1990; 76: 653–656.
3. Pan M *et al.* 6-Shogaol suppressed lipopolysaccharide-induced up-expression of iNOS and COX-2 in murine macrophages. *Mol Nutr Food Res* 2008; 52: 1467–1477.
4. Scott K. Ginger – powerful antioxidant for Alzheimer's, cancer and heart disease. 2007. http://ezinearticles.com/?Ginger---Powerful-Antioxidant-for-Alzheimers,-Cancer-and-Heart-Disease&id=773486 (Accessed on 10 January 2011).
5. Kaur IP *et al.* Entrapment of probiotic bacteria within alginate-HPMC floating beads, their survival and effectiveness against ethanol induced ulcers in rats. *Int J Probiotics Prebiotics* 2007; 2: 141–148.
6. Rao JM *et al.* Composition for treating gastric ulcer and a process for preparing the same. US patent 6855347 02/15/2005, 2005.
7. Singh PK, Kaur IP. Development and evaluation of a gastro-retentive delivery system for improved antiulcer activity of ginger extract (*Zingiber officinale*). *J Drug Target* 2011; 19: 741–751.
8. Ishak RAH *et al.* Preparation, in vitro and in vivo evaluation of stomach-specific metronidazole-loaded alginate beads as local anti-*Helicobacter pylori* therapy. *J Control Release* 2007; 119: 207–214.
9. Kasugai K *et al.* Crucial role of c-Jun NH2-terminal kinase 1 (JNK1) in cold-restraint stress-induced gastric lesions in mice. *Dig Dis Sci* 2007; 52: 1698–1705.
10. Goel RK *et al.* Oxidative stress and antioxidants status in peptic ulcer and gastric carcinoma. *Indian J Physiol Pharmacol* 2004; 48: 115–118.
11. Narayan S *et al.* Protective effect of a polyphenol drug, ambrex in ethanol induced gastric mucosal lesions in experimental rats. *Ind J Pharmacol* 2004; 36: 34–37.
12. Bilgin HM *et al.* Role of ghrelin in the regulation of gastric acid secretion involving nitrergic mechanisms in rats. *Physiol Res* 2007; 57: 563–568.
13. El-sokkarya GH *et al.* Effect of chronic nicotine administration on the rat lung and liver: beneficial role of melatonin. *Toxicology* 2007; 239: 60–67.
14. Cuppett SL *et al.* Hydrogen peroxide induced oxidative stress damage and antioxidant enzyme response in Caco-2 human colon cells. *J Agric Food Chem* 2005; 53: 8768–8774.
15. Schinella GR *et al.* Antioxidant effects of aqueous extract of *Ilex paraguariensis*. *Biochem Biophys Res Commun* 2000; 269: 357–360.
16. Gornall AG *et al.* Determination of serum proteins by means of the biuret reaction. *J Biol Chem* 1949; 177: 751–766.

17. Esposito P *et al*. Investigation of surface properties of some polymers by a thermodynamic and mechanical approach: possibility of predicting mucoadhesion and biocompatibility. *Biomaterials* 1994; 15: 177–182.
18. Tandon R *et al*. Oxidative stress and antioxidants status in peptic ulcer and gastric carcinoma. *Indian J Physiol Pharmacol* 2004; 48: 115–118.
19. Repetto MG, Llesuy SF. Antioxidant properties of natural compounds used in popular medicine for gastric ulcers. *Braz J Med Biol Res* 2002; 35: 523–534.
20. Ye YN *et al*. Effect of polysaccharides from *Angelica sinensis* on gastric ulcer healing. *Life Sci* 2003; 72: 925–932.
21. Bode C, Bode JC. Effect of alcohol consumption on the gut. *Best Pract Res Clin Gastroenterol* 2003; 17: 575–592.

