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Graphical abstract
Development of floating in-situ gelling system as an efficient anti-ulcer formulation: In-vitro and in-vivo studies

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ABSTRACT

Aim of the present work was to design gellan gum and calcium carbonate based floating in-situ gel as an efficient anti-ulcer formulation using andrographolide (AG) as a model drug. A $3^2$ factorial design was used to study the effect of gellan gum and calcium carbonate on characteristic of in-situ gelling system. The formulations were evaluated in terms of in-vitro, in-vivo anti-ulcer and histopathological study in Wistar rats. Drug content and viscosity were found in the range of $74.3 \pm 1.2$-$95.5 \pm 1.8\%$ and $67.7 \pm 1.6$ to $152.13 \pm 1.1$ cps, respectively. Formulation gelled within 2 sec and floated more than 24 hrs in simulated gastric fluid; an initial burst release of $11.7 \pm 0.9$ to $32.4 \pm 2.1\%$ till 1hr followed by a sustained release was observed. In-vivo, the AG floating in-situ gel (AGFIG) demonstrated lower acid and protein level, high haemoglobin level and negligible ulcer index. Moreover, it preserved integrity and histological aspects of the gastric mucosa as compared to pure AG and ranitidine. Improved anti-ulcer activity of AGFIG was attributed to longer residence time of AG in the stomach which improved the activity of myeloperoxidase, lipid peroxide, mucin content and glutathione peroxidise on gastric mucosal surface leading to protection from alcohol induced erosion. The study concluded that such floating in-situ gelling system can be translated for existing and established anti-ulcer drugs.
Introduction

Gastric ulcers are common pathologies that affect a significant number of people around the world.\textsuperscript{1} The increased incidence of gastric ulcers is associated with aggressive factors against the gastric mucosa such as ethanol exposure, stress, smoking, nutritional deficiencies and frequent ingestion of non-steroidal anti-inflammatory drugs.\textsuperscript{2,3} Although many conventional drugs are available to treat ulcers, most of these drugs have adverse reactions when used over long term.\textsuperscript{4} Moreover, short residence time of drug leads to incomplete eradication of gastric ulcer as there is insufficient concentration of the drug in the gastric mucous layer or epithelial cell surface. The instability of the drug in the low pH of gastric fluid can also be a reason for it.\textsuperscript{5} Therefore, it is necessary to design drug delivery systems that not only alleviate the shortcomings of conventional delivery system but also deliver the drug into the epithelial cells.

Different therapeutic strategies have been studied for complete eradication of the gastric ulcer. One way to improve the efficacy in eradicating the gastric ulcer is to deliver the drug locally in the stomach.\textsuperscript{6} Better stability and longer residence time (gastroretentive system) will allow more of the drug to contact the gastric mucus layer.\textsuperscript{7} Many approaches have been reported in the literature for the formulation of gastro retentive systems.\textsuperscript{8} Each approach has its own limitations. For example, ‘swelling and expanding systems’ may show a hazard of permanent retention. Bioadhesive systems may result in irritation of the mucous layer due to high localized concentration of the drug.\textsuperscript{9,10} In addition single-unit systems such as tablets or capsules may exhibit the all-or none emptying phenomenon leading to variability in bioavailability.\textsuperscript{11-13} Floating \textit{in-situ} gel (FIG) formulations present a novel and interesting approach to obtain gastro retentive sustained release of drugs and FIG has been developed for several drugs.\textsuperscript{14} This system
would have the advantage of ease of administration, as being a liquid and also be more patient compliant.

Natural products have gained powerful attention due to its effective role in chemotherapeutic agents and chronic disease prevention, including gastric ulcer. *Andrographis paniculata* Nees (Acanthaceae family) is an Indian herbal medicine.\textsuperscript{15,16} Recently, *A. Paniculata* leaves extract showed gastroprotective activities by mechanism of regulating pH, increase mucous production and anti-oxidant property.\textsuperscript{17} Diterpenoids and flavonoids are the primary constituents found in leaves of *A. Paniculata*, in particular, andrographolide (AG) is the major metabolites which is responsible for major biological activities.\textsuperscript{18,19} In the present study, AG was selected as a model drug to develop FIG as an efficient anti-ulcer formulation. Because, AG possesses similar physicochemical properties with many frontline anti-ulcer therapeutics such as high lipophilicity (log \(P\) 2.632 ± 0.135), insolubility in water (3.29 ± 0.73 µg/ml),\textsuperscript{19} site specific absorption from upper part of GI tract and short biological half life (4.6 hr).\textsuperscript{20} Such a formulation design challenges can be translated for existing and established drug candidates as well.

Gellan gum (commercially available as Gelrite or Kelcogel), a biomaterial, is an anionic deacetylated exocellular polysaccharide secreted by *Pseudomonas elodea* with tetrasaccharide repeating unit of one \(\alpha\)-L-rhamnose, one \(\beta\)-D-glucuronic acid and two \(\beta\)-D-glucose residue.\textsuperscript{21} Gellan has a characteristic temperature and ion dependent gelling property.\textsuperscript{22} Aqueous solution of gellan gum forms gel on warming to body temperature and in presence of cations by the mechanism of formation of double helical junction zones followed by aggregation of those double helical segments to form a three dimensional network by complexation with cations and hydrogen bonding with water.\textsuperscript{23,24} It has been used investigated for the controlled release of various kinds of drugs.\textsuperscript{25-28}
The basic strategy adopted in this study involved incorporation of calcium carbonate and sodium citrate in gellan gum-AG dispersion. Initially, the calcium carbonate is solubilised in the acidic environment of the stomach and the released calcium ions are then complexed by sodium citrate. However, a slow conversion of the complexed calcium into free calcium causes gelation of gellan, the gelled material floats upwards in the stomach with a potential to release its drug over a period of time. The calcium carbonate present in the formulation releases carbon dioxide in the gastric environment, thereby making the formulation buoyant and prolonging the residence time.

The aim of the present investigation was to design gellan gum based FIG drug delivery system as an efficient anti-ulcer formulation using AG as a model drug. The work undertaken included the preparation, optimization and evaluation of AG floating in situ gel (AGFIG). A $3^2$ factorial design assisted in the statistical optimization. The AGFIG was evaluated for drug content, viscosity, in-vitro gelation, in-vitro buoyancy, in-vitro drug release, in-vivo anti-ulcer and histopathological study in Wistar rats.

**Experimental section**

**Materials**

Andrographolide (AG) was purchased from Research Organic, Chennai, India. Gellan gum was purchased from Sigma Chemical Ltd (New Delhi, India). Sodium citrate and calcium carbonate was obtained from Shakti Chemicals, Mehsana, India. Ranitidine was a gift from Lupin Pharma Ltd, Pune, India. Methanol and ethanol GR grade were purchased from Merck Chemicals, Mumbai, India. All other materials and chemicals used were of either pharmaceutical or analytical grade.
Preparation of in-situ gelling solution

Gellan gum, at different concentrations (0.3–0.4 % w/v) was prepared in deionised water containing sodium citrate (0.25% w/v). Low level of cations present in the solution was sufficient to hold the molecular chains together and inhibit hydration. The gellan gum solution was heated to 90°C with stirring. After cooling below 40°C, various concentrations of calcium carbonate (0.5-1% w/v) and AG (0.1% w/v) solution in methanol were added and dispersed well with continuous stirring until to evaporate methanol. The resulting gellan gum in-situ gel solution containing AG (AGFIG) was finally stored in narrow mouth bottles until further use.

Factorial design

AGFIG system was prepared on the basis of $3^2$ factorial design where, amount of gellan gum ($X_1$) and calcium carbonate ($X_2$) were selected as two independent variables. Three levels determined from preliminary studies of each variable were selected and nine possible batches were prepared using different levels of variables (Table 1). The key factors selected during the optimization were viscosity, floating lag time and percentage drug release at 1 hr ($Q_{1h}$). The data analyses of values obtained from various batches were subjected to multiple regression analysis using PCP Disso software. The equation fitted was

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{12}X_1X_2$$

where $Y$ is the measured response; $X$ is the levels of factors; $\beta$ is the coefficient computed from the responses of the formulations.
Table 1 Coded levels and actual values of the variables along with the measured responses of $3^2$ factorial design

<table>
<thead>
<tr>
<th>Batch</th>
<th>Coded Levels</th>
<th>Gellan (%) w/v</th>
<th>CaCO$_3$ (%) w/v</th>
<th>Drug content (%)</th>
<th>Viscosity (Cps)</th>
<th>Floating lag time (Sec) at 1 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>–1, –1</td>
<td>0.3</td>
<td>0.5</td>
<td>85.5 ± 1.7</td>
<td>67.7 ± 1.6</td>
<td>5.44 ± 0.12</td>
</tr>
<tr>
<td>F2</td>
<td>–1, 0</td>
<td>0.3</td>
<td>0.75</td>
<td>91.2 ± 1.6</td>
<td>74.1 ± 1.3</td>
<td>4.55 ± 0.31</td>
</tr>
<tr>
<td>F3</td>
<td>–1, 1</td>
<td>0.3</td>
<td>1</td>
<td>88.7 ± 1.2</td>
<td>85.2 ± 1.8</td>
<td>2.22 ± 0.27</td>
</tr>
<tr>
<td>F4</td>
<td>0, –1</td>
<td>0.35</td>
<td>0.5</td>
<td>90.3 ± 1.3</td>
<td>95.3 ± 1.5</td>
<td>5.33 ± 0.18</td>
</tr>
<tr>
<td>F5</td>
<td>0, 0</td>
<td>0.35</td>
<td>0.75</td>
<td>95.5 ± 1.8</td>
<td>105.9 ± 1.6</td>
<td>4.52 ± 0.24</td>
</tr>
<tr>
<td>F6</td>
<td>0, 1</td>
<td>0.35</td>
<td>1</td>
<td>74.3 ± 1.2</td>
<td>115.9 ± 1.8</td>
<td>2.04 ± 0.33</td>
</tr>
<tr>
<td>F7</td>
<td>1, –1</td>
<td>0.4</td>
<td>0.5</td>
<td>82.3 ± 1.4</td>
<td>125.7 ± 1.7</td>
<td>4.77 ± 0.19</td>
</tr>
<tr>
<td>F8</td>
<td>1, 0</td>
<td>0.4</td>
<td>0.75</td>
<td>84.6 ± 1.1</td>
<td>137.1 ± 1.3</td>
<td>2.23 ± 0.19</td>
</tr>
<tr>
<td>F9</td>
<td>1, 1</td>
<td>0.4</td>
<td>1</td>
<td>86.5 ± 1.5</td>
<td>152.4 ± 1.1</td>
<td>2.10 ± 0.18</td>
</tr>
</tbody>
</table>

All the determinations were performed in triplicate and values are expressed as mean ± S.D., $n = 3$;

X1: Gellan gum; X2: Calcium carbonate.
Evaluation of AGFIG system

Measurement of density and viscosity. The relative density was measured by the mass by volume method. All measurements were made in triplicate. The viscosities of the prepared solutions were determined by Brookfield viscometer (Model LVDV-II Pro, USA). The samples (100 ml) were sheared at a rate of 100 r/min using suitable spindle at room temperature. Viscosity measurement for each sample was done in triplicate with each measurement taking approximately 30 s.29

Determination of drug content. Accurately, 10 mL of in-situ gelling solution was transferred to 100 mL volumetric flask. To this 50-70 mL of 0.1 N HCl was added and sonicated for 30 min. Volume was adjusted to 100 mL. Complete dispersion of contents was ensured visually and the dispersion was filtered using Whatman Filter Paper. From this solution, 10 mL of sample was withdrawn and diluted to 100 mL with 0.1 N HCl. Contents of AG was measured at maximum absorbance at 227 nm using UV-Visible Spectrophotometer at (V-630, Shimadzu Co Ltd., Japan). Percent drug content was determined using formula

\[
\text{Percent Drug Content} = \frac{\text{Actual Drug Content}}{\text{Total Drug Amount Taken}} \times 100
\]

In-vitro gelation study. Accurately measured 10 ml of AGFIG solution was added to 500 ml of simulated gastric fluid (pH 1.2) with mild agitation that avoids breaking of formed gel. Gelling was observed visually by qualitative measurement.30
**In-vitro floating study.** The in-vitro floating study was conducted in USP dissolution apparatus II containing 500 ml of simulated gastric fluid (pH 1.2) maintained at 37°C. A petri dish (4.5 mm internal diameter) containing 10 ml of AGFIG formulation was kept in the dissolution vessel containing medium without much disturbance. The time the formulation took to emerge on the surface of the dissolution medium (floating lag time) and the time the formulation constantly floated on the dissolution medium surface (duration of floating) were noted.31

**In-vitro drug release study.** The release of AG from AGFIG preparations was determined as described by Miyazaki et al.,32 with a slight modification that USP dissolution test apparatus (USP 24) with a paddle stirrer at 50 rpm was used. This speed was slow enough to avoid the breaking of gelled formulation and was maintained the mild agitation conditions believed to exist in-vivo. A petri dish (4.5 mm internal diameter) containing AGFIG formulation equivalent to 10 mg of AG was kept in the dissolution vessel containing simulated gastric fluid (pH 1.2) maintained at 37°C medium without much disturbance. During the dissolution, a precisely measured sample of the dissolution medium was removed at predetermined time intervals and replenished with pre warmed (37°C) fresh medium. Absorbance of AG was measured at 227 nm using UV spectrophotometer (Shimadzu, UV-1601, Japan). Each study was conducted in triplicate till 24 h. The Q1h (Cumulative amount of drug release at 1 hr) was calculated for all the formulations in order to find the effect of gellan gum and calcium carbonate on the release profile.
In-vivo anti-ulcer activity in rats

Animals. The anti-ulcer activity of AGFIG solution was studied on ethanol induced gastric ulcer rats in comparison with pure AG and standard ranitidine.\textsuperscript{33-35} Thirty male Wistar albino rats, 200-250 g were obtained from Lachmi farm, Alephata, Pune, India. The protocol of the experiment was approved by Institutional Animal Ethics Committee of Sharadchandra Pawar College of Pharmacy (Otur, Pune, India). The rats were housed in polypropylene cages with free access to standard laboratory diet and water. They were kept at 25 ± 1°C and 45–55% RH with a 12-h light–dark cycle. The animals were fasted, but provided free access to water, overnight before the commencement of the experiment.

Experimental design and treatments. The study was performed using a randomized design with rats randomly divided into five groups (six animals in each group).

Group 1 received distilled water orally for 14 consecutive days followed by fasting on last day.

After 24 hrs fasting, rats received normal saline (15 ml/kg) orally and served as a control group.

Group 2 received distilled water orally for 14 consecutive days followed by fasting on last day.

After 24 hrs fasting, rats received 1 ml ethanol (80%) orally and served as the toxic group.

Group 3 received AGFIG formulation equivalent to 10 mg/kg of AG for 14 consecutive days followed by fasting on last day. After 24 hrs fasting, rats received 1 ml ethanol (80%) orally.

AGFIG contains calcium carbonate and sodium citrate in gellan gum-AG dispersion. Initially, the calcium carbonate soluble in the acidic environment of the stomach and the released
calcium ions then are complexed by the sodium citrate. However, a slow conversion of the complexed calcium into free calcium causes gelation of gellan, the gelled material floats upwards in the stomach with a potential to release its drug over a period of time. The calcium carbonate present in the formulation releases carbon dioxide in the gastric environment, thereby making the formulation buoyant and prolonging the residence time.\textsuperscript{36}

Group 4 received pure AG (10 mg/kg suspended in 0.3% carboxymethyl cellulose) for 14 consecutive days followed by fasting on last day. After 24 hrs fasting, rats received 1 ml ethanol (80\%) orally.

Group 5 received ranitidine (30 mg/kg suspended in 0.3% carboxymethyl cellulose) orally for 14 consecutive days followed by fasting on last day. After 24 hrs fasting, rats received 1 ml ethanol (80\%) orally.

**Determination of hemoglobin and basal gastric acid secretion.** Before and after 4 h of final treatment, the blood samples collected with the anticoagulant were used immediately for the determination of hemoglobin using a veterinary blood cell counter (ERMA Inc., PCE 210 Vet). After collecting blood samples, rats were anesthetized; a sharp blow on the head sacrificed the animals. After sacrifice, the abdominal wall was incised longitudinally and the stomach was isolated from the surrounding tissues by means of two cuts. The first was done 1 cm proximal to the cardiac sphincter followed by injecting saline solution through the esophageal junction (Fig. 1a) and the second cut made at 1 cm distal to the pyloric sphincter (Fig. 1b). The stomach was removed and both ends were tied by means of threads. The stomach was kept immersed in the saline solution in a petri plate for 30 min. After 30 min, the threads were removed and saline solution was drained from the stomach by simply squeezing the stomach so that the fluid could
leave the stomach through the esophageal junction. The gastric fluid collected in this manner was assessed for volume of gastric content, pH, acidity and bovine albumin.

**Fig. 1** Collection of gastric content for determination of basal gastric acid secretion for AGFIG (andrographolide floating *in-situ* gel) in rat stomach.
**Determination of pH and total acidity.** The collected gastric content was centrifuged at 1000×g for 10 min to remove residual debris. Net volume of gastric fluid was measured, pH was determined by pH meter and total acidity was determined by titration against 0.01N NaOH to pH 7 and expressed as meq/l/100. Acidity was calculated by the following formula.

\[
\text{Acidity} = \frac{\text{Volume of NaOH} \times \text{Normality of NaOH}}{0.1} \times 100 \text{(meq/l/100g)}
\]

**Estimation of bovine serum albumin.** A 9 ml of 90% alcohol was added into 1 ml of gastric juice in order to precipitate dissolved proteins in gastric juice. The alcoholic precipitate was dissolved in 1ml of 0.1N NaOH. 0.05 ml NaOH transferred into a test tube to which 4 ml of alkaline mixture was added and kept for 10 min, then 0.4 ml of phenol reagent was added and kept for 10 min to develop colour. Absorbance was taken against blank with distilled water at 610 nm using spectrophotometer. A calibration curve was prepared with a range of bovine albumin concentration from 2 to 10 µg/ml in water at 610 nm. The amount of bovine serum albumin content was calculated according to the calibration curve equation \( y = 0.0071x - 0.0102, R^2 = 0.996 \). It has been expressed in terms of µg/ml of gastric juice.

**Determination of gastric lesion index.** After gastric fluid collection, the stomach was opened along the greater curvature; the presence of blood or mucus was noted and then rinsed gently under running tap water. The stomach was then placed on a petri dish and examined for gastric lesion index. The ulcerative lesion index of each animal was calculated according to Gamberini et al. and scored as follows: loss of mucosal folding, mucosal discoloration, edema.
or hemorrhage (score 1 each); ulcers/cm² less than 1 mm (score – number of ulcer ×2); ulcers more than 1 mm/cm² (score – number ×3); perforated ulcers (score – number × 4).

**Histopathological examination.** For the histopathological examination, the stomach tissue samples were fixed in 10% formaldehyde and processed routinely for embedding in paraffin. Paraffin sections were stained with haematoxylin and eosin to indicate histological degeneration, these were examined under microscope (100X) for histopathological changes such as ulceration, hyperemia, necrosis, edema, cellular infiltration and atrophy of villi. The severity of histopathological changes was expressed according to an arbitrary scale as follows 0 (normal), + (mild), ++ (moderate), +++ (severe).

**Results and discussion**

AG, a naturally occurring bioactive phytoconstituent, possesses a wide range of pharmacological activities and medicinal properties. However, because of site specific absorption from upper part of GI tract, pH-dependent hydrolysis in neutral or weak alkaline environment of intestine and sulfonated phase I metabolism in the small intestine, it has limited bioavailability. The concept of *in-situ* floating system using gellan gum was applied for delivery of AG in order to enhance its therapeutic efficacy.

**Evaluation of formulation**

The two main pre-requisites of *in-situ* gelling systems are optimum viscosity and gelling capacity. The formulation should have an optimum viscosity that will allow easy swallowing as a liquid, which then undergoes a rapid sol-gel transition due to ionic interaction. Moreover, the *in-
in-situ formed gel should preserve its integrity without dissolving or eroding for prolonged period to facilitate sustained release of drug locally. The developed formulations met all prerequisites to become an *in-situ* gelling floating system, gelled and floated instantaneously in the pH conditions of the stomach (Fig. 2).\textsuperscript{29} Density is important parameter as far as the floating properties of the gastro retentive dosage form is concerned. Ideally density of the dosage form to float on the gastric content must have density less than or equal to gastric content (1.004 g/cm\textsuperscript{3}). The relative density values of the batches were 0.8976 g/cm\textsuperscript{3} – 0.9840 g/cm\textsuperscript{3}, values favorable for floating. The drug content ranged from 74.3 ± 1.2-95.5 ± 1.8% indicating homogenous distribution of drug throughout *in-situ* gel solution.

![Image](image_url)

**Fig. 2** Photograph showing the formation of gellan gel in simulated gastric fluid pH 1.2.

Sol to gel transformation of gellan occurs in the presence of either monovalent or divalent cations in contact with the gastric fluids. The calcium carbonate present in the formulation as insoluble dispersion dissolves and releases carbon dioxide and calcium ions on reaction with acid
resulting in formation of a floating gel. The released carbon dioxide is entrapped in the gel network of the formulation and the gel rises to surface of the dissolution medium or stomach.\textsuperscript{6} It is established that formulation containing calcium carbonate produces a significant stronger gel than those containing sodium carbonate.\textsuperscript{40} This is due to the internal ionotropic gelation effect of calcium on gellan.\textsuperscript{41}

\textit{In-vitro} gelation study

The gelation study was conducted in simulated gastric fluid (SGF, pH 1.2). All the formulation showed immediate gelation when contacted with the SGF. All the formulations gelled within 2 sec and the gelling time was ranged from 2 sec to 5.4 sec. The formulation containing highest concentration of calcium carbonate (1\% w/v) had shortest gelation time (2 sec), whereas formulation containing lowest calcium concentration had longest gelation time (5 sec). This again points to ion dependent gelation of gellan gum.

\textit{In-situ} formulations with low content of calcium carbonate (0.5\% w/v) formed weak gels. Such vehicles are not suitable as oral liquid formulations; as they will be removed earlier form the stomach by the peristaltic movements as compared to the rigid gel formed at the high concentration of calcium carbonate (1\% w/v) with a short gelation time. The optimum level of polymer and calcium carbonate demonstrated adequate gel strength when pressed with a pair of fine forceps, indicating that they will withstand the shear forces likely to be encountered in the stomach. Thus, such vehicles, although free flowing initially to allow reproducible oral administration, also provide longer residence time.
Optimization of AG \textit{in-situ} gel by $3^2$ factorial design

Many studies have reported that the major factors influencing the viscosity, floating lag time and drug release of floating gel are interaction between the drug and polymers. During preliminary study, the concentrations of gellan gum ($X_1$) and calcium carbonate ($X_2$) which would give a non-aggregating, non-sedimenting gel were determined. The concentrations of gellan gum ($X_1$) and calcium carbonate ($X_2$) were predicted to be decisive in the preparation and stabilization of the in-situ gel system. A $3^2$ factorial design was employed to optimize their concentrations (Table 1). AG concentration was kept constant.

As per $3^2$ factorial design, nine different batches were prepared. The responses of these batches are shown in Table 1. The data obtained was subjected to multiple regression analysis using ‘PCP Disso V3’ software (IIPC, PCP, Pune, India) and fitted with the equation:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{12}X_1X_2$$

Where $Y$: measured response; $X$: levels of factors; $\beta$: coefficient computed from the responses of the formulations.

The results of multiple regression analysis of the obtained data were shown in Table 1. The adequacy of fitted model was checked by analysis of variance (ANOVA). To study the interaction effects of the independent variables, response surface plots were constructed using ‘PCP Disso V3’ software (IIPC, PCP).
Fig. 3 Response surface plot showing effect of factorial variables on viscosity, floating lag time and percentage drug release at 1 hr.
**Effect on viscosity.** The rheological properties of the solution are of importance in view of their proposed oral administration because it affects the pourability, rate of gelation of solution and time required for the gelation. In the selection of the concentration of the gelling polymer, a compromise is sought between concentrations that can form satisfactory gel strength as a delivery vehicle and viscosity for ease of administration. The effect of gellan concentration on viscosity was studied using $3^2$ factorial design and the results of multiple regression analysis for viscosity as follows

$$\text{Viscosity (cps)} = 106.25 + 31.86X_1 + 10.30X_2, \quad R^2, 0.9938$$

The viscosity was in the range of $67.7 \pm 1.6$ to $152.13 \pm 1.1$ cps (Table 1) and was strongly influenced by the independent variables. As shown in the surface plot (Fig. 3), viscosity increased with increasing concentrations of gellan gum which may be due higher chain interactions within the polymer. Increasing the calcium carbonate content in the formulation simultaneously increased the viscosity at all the polymer concentrations studied. Increase in dispersed insoluble particles of calcium carbonate in formulation also contributing to viscosity.

**Effect on floating lag time.** The floating ability of the prepared formulations in terms of floating lag time and duration of floating was evaluated in SGF pH 1.2. All formulations showed floating for more than 24 h whereas the floating lag times were varied (Table 1). Upon contact with an acidic medium, calcium carbonate effervesced, releasing carbon dioxide and calcium ions. The cross-linking by Ca$^{++}$ ions and gellan provides barrier effect at the surface of the formulation. The released carbon dioxide entrapped in the gel network producing buoyant formulation. Then, calcium react with gellan produce a cross linked three-dimensional gel network restrict further diffusion of carbon dioxide as well as drug and result in extended
duration of floating and drug release.\textsuperscript{29} The floating ability of the formulation was primarily dependent on calcium carbonate concentrations. The results of multiple regression analysis for floating lag time is as follows

\[ F_{\text{lag}} \text{ (sec)} = 3.66 - 1.53X_2, \quad R^2 = 0.7897 \]

The floating lag time was in the range of 3 to 5 sec and was strongly influenced by the independent variables. As shown in surface plot (Fig. 3), increasing the calcium carbonate concentration reduced the floating lag time and extended duration of floating. This is probably related to increase in the amount of Ca\textsuperscript{++} and CO\textsubscript{2} content.\textsuperscript{42}

**Effect on in-vitro release.** The cumulative percentage release at 1h (Q\textsubscript{1h}) is indicated in Table 1. The effect of polymer concentration on Q\textsubscript{1h} is shown in Fig.4 and multiple regression analysis reveals the following relation

\[ Q_{1h} = 22.66 - 6.25X_1, \quad R^2 = 0.7464 \]

A significant decrease in the rate and extent of drug release was observed with the increase in polymer concentration. It was attributed to increase in the density of the polymer matrix and the consequent increase in the diffusional path length across which the drug molecules have to traverse. The release of drug from these gels was characterized by an initial phase of burst release. However, as gelation proceeds, the remaining drug was released moderately in the second phase. This bi-phasic pattern of release is a characteristic feature of matrix diffusion kinetics.\textsuperscript{43} The initial burst effect was considerably reduced with increase in polymer concentration. From the factorial design, results revealed that the batch 5 (F5) demonstrated acceptable viscosity, floating lag time and Q\textsubscript{1h}, it was deemed as the optimized formulation and subjected to further in-vivo anti-ulcer study in wistar rats.
Fig. 4 *In-vitro* drug release profiles of AG (andrographolide) from AGFIG (andrographolide floating *in-situ* gel) formulations.
In-vivo study

*Estimation of gastric pH, acidity and bovine serum albumin.* As shown in Table 2, all the groups showed acidic gastric pH. However, Pure AG, AGFIG and ranitidine showed higher pH as compared to toxic and control groups. The lower acidity and protein levels of group treated with AGFIG as compared to pure AG and ranitidine indicated potent anti-ulcer activity of AGFIG.
Table 2  Effect of formulations on volume of gastric content, pH, acidity, serum albumin content and hemoglobin

<table>
<thead>
<tr>
<th>Groups</th>
<th>Volume of gastric content (ml)</th>
<th>pH</th>
<th>Acidity</th>
<th>Serum albumin content (µg/ml)</th>
<th>Hb before treatment</th>
<th>Hb after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.2 ± 0.6</td>
<td>2.36 ± 0.8</td>
<td>21 ± 3</td>
<td>115.6 ± 5.3</td>
<td>12.8 ± 0.8</td>
<td>12.2 ± 0.3</td>
</tr>
<tr>
<td>Toxic</td>
<td>1.1 ± 0.4</td>
<td>1.81 ± 0.4</td>
<td>36 ± 5</td>
<td>144.6 ± 7.1</td>
<td>12.5 ± 0.3</td>
<td>6.1 ± 0.4</td>
</tr>
<tr>
<td>Pure AG</td>
<td>1.5 ± 0.2</td>
<td>3.42 ± 0.9</td>
<td>16 ± 2</td>
<td>113.9 ± 3.2</td>
<td>11.6 ± 0.6</td>
<td>6.3 ± 0.5</td>
</tr>
<tr>
<td>AGFIG (F5)</td>
<td>1.6 ± 0.3</td>
<td>3.48 ± 0.5</td>
<td>12 ± 2</td>
<td>84.6 ± 2.4</td>
<td>12.1 ± 0.2</td>
<td>9.4 ± 0.4</td>
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<tr>
<td>STD RAN</td>
<td>1.3 ± 0.4</td>
<td>3.45 ± 0.7</td>
<td>15 ± 2</td>
<td>112.5 ± 4.6</td>
<td>12.1 ± 0.2</td>
<td>7.1 ± 0.6</td>
</tr>
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</table>

Hb hemoglobin, AG andrographolide, AGFIG andrographolide floating in-situ gel, STD RAN standard ranitidine.

All the determinations were performed in triplicate and values are expressed as mean ± S.D., n = 3.
**Fig. 5** Effect of formulations on gross appearances of stomach. Control (a), 80% ethanol (b), pure AG (C), AGFIG (c) and ranitidine (d). AG andrographolide, AGIFG andrographolide floating *in-situ* gel.
Pre and post treatment, the haemoglobin levels were evaluated in all the groups. No significant rise in pre-treatment haemoglobin levels was observed in all groups. However, in post-treatment, significant increase in haemoglobin level was observed for AGFIG formulation as compared to ranitidine and pure AG. These observations again supported potential prevention of GI ulceration and haemorrhage by AGFIG.

**Gross appearances of stomach and gastric ulcer index.** The animals that received 80% ethanol developed a consistent macroscopic damage as evidenced by presence of hemorrhagic ulceration (Fig. 5b). Ethanol produces a marked contraction of circular muscles of rat fundic strip. Such contraction may lead to mucosal compression at the site of greatest mechanical stress and crests of mucosal folds leading to necrosis and ulceration.\(^{35}\) It was attenuated by the prior administration of pure AG (10 mg/kg) as indicated by presence of few fields of ulceration hemorrhagic and hyperemia (Fig. 5c). The animals treated with the AGFIG formulation (10 mg/kg) and ranitidine (30 mg/kg) did not show any macroscopic toxicity (Fig. 5d and e), preserving the morphological integrity of the gastric mucosa similar to non-treated control group (Fig. 5a). Thus, AGFIG could prevent the ulcerative damage induced by ethanol.

In addition, the administration of ethanol induced a significant increase of ulcer index in relation to control animals (Table 3). However, the groups treated with pure AG showed a significant low ulcer index compared to animals treated with ethanol. Moreover, the animals that received AGFIG formulation and ranitidine did not show significantly different ulcer index compared to control animals. The improved anti ulcer activity of AGFIG can be attributed to longer residence time of AG in the stomach which improved the activity of myeloperoxidase, lipid peroxide, mucin content and glutathione peroxidise on gastric mucosal surface leads to
protection of surface from single dose of 80% alcohol induced erosion. The free drug could not
give such enhanced activity due to short residence in stomach.\textsuperscript{44}
<table>
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<tr>
<th>Groups</th>
<th>No of ulcer/ stomach (a)</th>
<th>Severity/ stomach (b)</th>
<th>Lesion index (a+b)</th>
<th>Ulceration</th>
<th>Hyperemia</th>
<th>Necrosis</th>
<th>Edema</th>
<th>Cellular infiltration</th>
<th>Atrophy of villi</th>
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</thead>
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<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>No ulcer seen</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
</tr>
<tr>
<td>Toxic</td>
<td>8.2 ± 0.9</td>
<td>8.0 ± 0.9</td>
<td>16.2 ± 1.8</td>
<td>+++</td>
<td>++++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Pure AG</td>
<td>3.3 ± 0.6</td>
<td>4.1 ± 0.5</td>
<td>7.4 ± 1.1</td>
<td>++</td>
<td>++</td>
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<td>++</td>
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<td>00</td>
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</tr>
<tr>
<td>STD RAN</td>
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<td>0</td>
<td>0</td>
<td>+</td>
<td>00</td>
<td>+</td>
<td>00</td>
<td>+</td>
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</tr>
</tbody>
</table>

AG andrographolide; F5 optimized AGFIG formulation; STD RAN standard ranitidine; Grades 0 (normal), + (mild), ++ (moderate), +++ (severe).
Fig. 6 Histopathology analysis of stomach from control (a), 80% ethanol (b), pure AG (C), AGFIG (d) and ranitidine (e). Photograph showing cellular infiltration (blue arrow), hemorrhages and congestion (red arrow), edema (white arrow), necrosis (black arrow) ulceration (yellow arrow). AG andrographolide; AGFIG andrographolide floating in-situ gel.

**Histopathology.** As compared to control group animals (Fig. 6a), the administration of 80% ethanol induced consistent microscopic damage with the presence of severe cellular
infiltration, hemorrhage and congestion, edema, necrosis, ulceration and atrophy of villi (Fig. 6b). The group which received pure AG presented minor hemorrhage and congestion, necrosis, ulceration and atrophy of villi caused by the aggressive action of ethanol, as a characteristic small inflammation but without the presence of inflammatory cells (Fig. 6c). Whereas, the gastric mucosa of animals which received AGFIG was completely protected against the ethanol action, preserving all histological aspects (Fig. 6d) when compared to control animals. Furthermore, the ranitidine group showed a relative protection against of ethanol with mild cellular infiltration, necrosis and ulceration (Fig. 6e).

**Conclusion**

A floating *in-situ* gelling system was developed as an efficient anti-ulcer formulation using AG as a model drug. A $3^2$ factorial design provided an optimized composition of the system. AGFIG formed a gel in stomach and sustained the drug release over a period of at least 24 h. It provided a site specific delivery of andrographolide for 24 hr. The *in-vivo* examination confirmed the enhanced efficacy of AGFIG in the treatment of gastric ulcer as compared to pure AG. Such floating *in-situ* gelling system can be translated for existing and established anti-ulcer drug as well as formulations. Developed systems can reduce the dosing regimen, increase patient compliance and further enhance the therapeutic efficacy of anti-ulcer drugs.

**Disclosure of interest**

The authors declare that they have no conflicts of interest concerning this article.
Notes and references


