

Evaluating the Mucoprotective Effects of Glycyrrhizic Acid Loaded Polymeric Nanoparticles Against 5-Fluorouracil Induced Intestinal Mucositis in Murine Model via Suppression of Inflammatory Mediators and Oxidative Stress

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Abstract

Objectives: 5-Flourouracil (5-FU), a chemotherapeutic drug, is linked with severe deteriorating effects on intestine leading to mucositis. Further, Glycyrrhizic acid is a renown herbal medicine with combined mucoprotective, antioxidant and anti-inflammatory actions, however associated with pharmacokinetics limitations. Owing to its remarkable therapeutic action in inflammatory bowel disease inside the polymeric nanocarriers, we have tried to explore its activity against 5-FU led intestinal mucositis. Polymeric nanocarriers proved to be efficient drug delivery vehicles for long-term remedy against inflammatory diseases, however, yet not explored for 5-FU induced mucositis. Therefore, the study aimed to produce Glycyrrhizic acid loaded poly lactic-co-glycolic acid (GA-PLGA) nanoparticles to evaluate its protective and therapeutic effects on intestinal mucosa against 5-FU mediated mucositis.

Methods: For the said purpose, GA-PLGA nanoparticles were prepared using modified double emulsion method, physicochemically characterized and tested for invitro drug release. Thereafter, mucositis was induced by 5-FU (50 mg/kg; IP) administration to the mice for the first three days (day 0, 1, 2) and orally treated with GA-PLGA nanoparticles till seventh day (day 0-6).

Results: GA-PLGA nanoparticles significantly reduced mucositis severity as manifested through recovered body weight, diarrhea score, distress, and anorexia. Further, 5-FU induced intestinal histopathological damage, altered villi-crypt length, low goblet cell count, elevated pro-inflammatory mediators and suppressed antioxidant enzymes were reversed by GA-PLGA nanoparticles' sustained release therapeutic action.

Conclusion: Morphological, behavioral, histological, and biochemical results suggested that GA-PLGA nanoparticles found to be efficient, biocompatible, targeted, sustained release drug delivery nano-vehicle for enhanced mucoprotective, anti-inflammatory and antioxidant effects in ameliorating 5-FU intestine mucositis.

1. Introduction

Intestinal mucositis is clinically characterized through severe abdominal pain, nausea, vomiting, bloating, diarrhea, and abdominal cramps because of underlying mucosal inflammation and loss of epithelial lining that ends up in mucosal ulceration of the gastrointestinal tract (Blijlevens et al. 2000; Logan et al. 2009). It is one of the inevitable side effects associated with cancer chemotherapy (van Vliet et al. 2010), affecting almost 40% of the patients receiving standard doses and about 80-100% of the patients who received higher doses of chemotherapy (Keefe et al. 1997; Lalla et al. 2014). Chemotherapeutic agents, in general, interfere with rapidly dividing cells, therefore, fast proliferating mucosal cells are more prone to these agents (Duncan and Grant 2003). Intestinal mucositis halt chemotherapeutic regimen and alter the dosing schedule, thereby contributing towards higher mortality in the cancer patients (Naidu et al. 2004).

Recently, research paradigm concentrates on the resolution of intestinal injury caused by chemotherapeutic agents. 5-Fluorouracil (5-FU) is a renown anti-cancerous drug, used to treat various

tumors such as head and neck, gastrointestinal and breast tumors. 5-FU, a fluorinated pyrimidine analogue, exhibited its anti-metabolite chemotherapeutic action through inhibition of DNA synthesis, however, it can induce intestinal mucositis through intestinal epithelial disruptions and indiscriminate action against rapidly dividing, fast proliferating intestinal mucosal cells which have increased sensitivity to 5-FU (Soares et al. 2008; Wright et al. 2009). Although complete pathogenesis of 5-FU induced intestinal mucositis is not fully understood, however, main stages of mucosal barrier damage include initial inflammatory phase when DNA strands breakage occurs, characterized through production of reactive oxygen species (ROS) with minimal disruption to the epithelial lining. The second stage involves excessive burst of pro-inflammatory cytokines (IL-1, IL-6, TNF- α , IFN- γ) and transcription factors (NF- κ B). It leads to epithelial phase where cells proliferation terminates and cell death begins, then progressed towards ulcerative phase consisting of cell necrosis and ulceration which is followed by the final healing phase that involves repair of damaged epithelial lining (Basile et al. 2019; Blijlevens et al. 2000). The five staged events heavily distress mucosal cells and causes intestinal villi-crypt atrophy and damage through facilitating the process of apoptosis, and burdened cells with oxidative and pro-inflammatory loads (Atiq et al. 2019; Basile et al. 2019).

Unfortunately, the synthetic therapy available for 5-FU induced mucositis itself associated with deleterious side effects (Atiq et al. 2019). Currently, research has been directed towards exploring the medicinal uses of herbal products for a range of ailments to avoid unnecessary effects associated with synthetic compounds (Yuan et al. 2016). In this context, Glycyrrhizic acid, a triterpene glycoside, obtained from the roots of licorice plants, *Glycyrrhiza glabra*, holds a vital position because of its various medicinal benefits including anti-inflammatory, anti-diabetic, antioxidant, anti-tumor, antimicrobial and anti-viral effects (Ming and Yin 2013). Recently, it has been explored that Glycyrrhizic acid possesses anti-inflammatory activity through inhibition of cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE-2) (Wang et al. 2011; Wang et al. 2017). Moreover, it can suppress inflammatory response through blocking NF- κ B pathway, which is responsible for the production of pro-inflammatory cytokines and chemokines (Cheng et al. 2006; Wang et al. 2017). Further, it has been found to possess anti-cancerous properties enabling its dual usage including prevention of chemo-induced mucosal damage and proliferation of tumorous lesions (Khan et al. 2013; Su et al. 2017). The potential of Glycyrrhizic acid has been explored where it has reduced tumorous mass development and prevented infiltration of mast cells with attenuation of TNF- α level and prevent mucosal layer disruptions (Khan et al. 2013; Khan et al. 2018). Furthermore, it was demonstrated that Glycyrrhizic acid owing to its anti-inflammatory and antioxidant properties have potential to serve as chemo-preventive agent (Bode and Dong 2015). Therefore, in the present study, Glycyrrhizic acid is explored to combat 5-FU induced intestinal mucositis and to highlight its anti-inflammatory, antioxidant, and muco-protective potential.

However, conventional delivery of Glycyrrhizic acid via oral route undergoes extensive first-pass effect resulting in decreased bioavailability and reduced therapeutic efficiency in medical conditions like intestinal mucositis (Ploeger et al. 2000). Therefore, a drug-delivery system is required to deliver the therapeutic agent to the inflamed intestines for an adequate duration without being metabolized in the gut. In this context, nanoparticle-based drug delivery system shows promising advantages to overcome

conventional therapy limitations due to their nano-range size that ultimately results in improved accumulation and enhanced residence time at the inflamed tissue via enhanced epithelial permeability and retention effect (eEPR) (Boisseau and Loubaton 2011; Zeeshan et al. 2019a). Previously, nanoparticles below 500 nm proved to be efficiently up taken by the infiltrated immune cells (Zeeshan et al. 2019a), which improves the time span for the drug release and therapeutic efficacy at the inflamed tissues while overcomes diarrhea led clearance. For nanoparticle mediated drug delivery, a large range of polymers can be employed, however, polymers such as, poly lactic-co-glycolic acid (PLGA) hold a crucial role due to their biodegradability, biocompatibility and their ability to attain a sustained drug release over a longer period to achieve better therapeutic outcomes (Danhier et al. 2012). In our previously reported work, PLGA nanoparticles have been shown remarkable therapeutic efficacy in ulcerative colitis model (Ali et al. 2016; Zeeshan et al. 2019b).

To date, PLGA nanocarriers were employed only to cure oral mucositis (Takeuchi et al. 2018), however, no evidence is available to establish their role in 5-FU induced intestinal mucositis. In the present study, 5-FU induced intestinal mucositis is developed in BALB/c mice and treated with both Glycyrrhizic acid free drug and Glycyrrhizic acid loaded PLGA (GA-PLGA) nanoparticles separately through oral administration to demonstrate the therapeutic efficacy of the drug with or without its nanocarrier against 5-FU mediated intestinal toxicity through evaluation of multiple morphological, behavioral, biochemical, and histological manifestations.

2. Materials And Methods

2.1 Preparation of GA-PLGA nanoparticles

GA-PLGA nanoparticles were prepared by modified double W/O/W emulsification-evaporation method, as reported previously (Zeeshan et al. 2019b). Glycyrrhizic acid (monoammonium salt), purchased from Sigma Aldrich, Germany, was dissolved in 5mL of distilled water and added dropwise in 5mL of organic solution, containing 100 mg PLGA (Resomer RG502, 50:50, Evonik, Germany) in 5 mL ethyl acetate. The resultant primary emulsion was probe sonicated and added into external aqueous phase, comprising 5mL of 2% poly vinyl alcohol (PVA) solution (Sigma Aldrich. Germany). The obtained W/O/W emulsion was stirred to evaporate organic solvent, centrifuged, and freeze-dried to get lyophilized Glycyrrhizic acid loaded PLGA nanoparticles.

2.2 Physicochemical characterization

Average particle diameter was determined through dynamic light scattering technique (Brookhaven 90Plus Instrument, USA) in triplicate and results are expressed in mean \pm standard deviation (S.D.). Zeta potential of nanoparticles was analyzed in triplicate using Malvern zeta sizer 2000 HS (Malvern instrument, UK) and stated as mean \pm S.D. The percentage yield of the freeze-dried nanoparticles was obtained by weighing freeze-dried nanoparticles and calculated by the formula:

$$\% \text{ Yield} = \frac{\text{Weight of freeze - dried nanoparticles}}{\text{Weight of polymer + drug}} \times 100 \quad \dots (1)$$

The surface morphology of GA-PLGA nanoparticles was analyzed under scanning electron microscope (SEM) (Vega 3 TESCAN, Czech Republic).

2.3 Encapsulation efficiency (EE)

Drug entrapment within PLGA nanoparticles was determined through the spectroscopic estimation of free drug content in the supernatant collected after centrifugation of the nano-formulation at a wavelength (λ_{max}) of 252 nm (Ultraviolet-Visible Spectrophotometer, Agilent Technologies, US). %EE was measured through the formula:

$$\% \text{ Encapsulation} = \frac{(\text{Total drug} - \text{Free drug})}{\text{Total drug}} \times 100 \quad \dots (2)$$

Afterwards, loaded drug content was determined spectroscopically at 252 nm by the method previously described (Zeeshan et al. 2019b). % Drug content was calculated from the formula:

$$\% \text{ Drug Content} = \frac{\text{Actual drug content in NPs}}{\text{Theoretical drug content used in formulation}} \times 100 \quad \dots (3)$$

2.4 Fourier transformed infrared spectroscopy (FTIR)

FTIR analysis of Glycyrrhizic acid drug, PLGA, and GA-PLGA nanoparticles was performed using potassium bromide (KBr) disc technique. FTIR spectra were observed in the region of 400–4000 cm^{-1} wave number against % transmittance using Perkin-Elmer100 FTIR spectrometer (US).

2.5 In-vitro drug release studies

In-vitro drug release was tested at pH 1.2 and 6.8 that corresponds to the physiological pH values of gastrointestinal tract (GIT) organs. 50 mg of the GA-PLGA nanoparticles were suspended in simulated gastric fluid (SGF), prepared according to United States Pharmacopeia. The release profile was observed for 2 hours, afterward salts $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ and KH_2PO_4 were added and pH was adjusted to 6.8 with 0.1N NaOH solution. The release profile was continued for 48 hours and the samples were collected at the pre-determined intervals from the buffers and analyzed under UV-Vis spectrophotometer for the drug release estimation.

2.6 Animal studies

2.6.1 Experimental Animals

Experiments were performed on 3–4 weeks old BALB/c mice obtained from National Institute of Health (NIH, Islamabad, Pakistan) and kept under standard laboratory condition (20–25°C, 55 ± 5% humidity) in stainless steel cages. Mice were acclimatized in the same environment for a period of one week before experimentation.

All animal experiments were conducted according to the bioethical committee protocols of Quaid-i-Azam University, Islamabad for the care and use of lab animals (Approval no.

BES-fbs-QAU2018-75). Mice were given ad libitum access to food and drinking water.

2.6.2 Induction and evaluation of mucositis

Mice weighing 25–30 g were randomly divided into four groups (n = 5/group). Number of mice in each group was decided while keeping in mind the ethical condition of minimal harm and applying resource equation to generate sound statistical data. Group one received vehicle only (drinking water) and served as a normal control group. Group 2, 3 and 4 received 5-FU (Sigma Aldrich, Germany) through intraperitoneal injection at a dose of 50 mg/kg for three consecutive days to induce experimental intestinal mucositis (Atiq et al. 2019). In addition, group 3 and 4 were treated with Glycyrrhizic acid free drug and GA-PLGA nanoparticles respectively through oral gavage, at a drug dose equivalent to 10mg/kg body weight for 7 days. The dose was optimized for anti-inflammatory activity in ulcerative colitis model in our previous study (Zeeshan et al. 2019b). For the first three days, treatment either in the form of free drugs or nanoparticles was given thirty minutes before 5-FU IP administration. At the 7th day, mice were euthanized, and tissue samples were removed for further testing (Fig. 1).

2.6.3 Assessment of intestinal mucositis severity through morphological and behavioral parameters

Mice were monitored to assess 5-FU induced mucositis severity on daily basis. It was manifested through body weight loss, stool consistency, stress scores, anorexia and mortality rate (Basile et al. 2019).

Therefore, mice from all four groups were observed daily for the body weight loss. The results were expressed as percent average body weight loss ± standard deviations (SD). Similarly, stools were examined for diarrheal indication and scored using Bowen's score system (Leocádio et al. 2015; Yeung et al. 2015).

Likewise, daily distress scores were calculated based on cumulative average scores of fur/coat condition, change in temperament and reluctance to movement. Fur/coat was characterized as 0, shiny and smooth; 1, coat raised around neck; 2, coat raised around neck and belly; 3, coat raised around whole body with or without coat loss. Change in temperament was noticed and scored as 0, normal; 1, agitated behavior; 2, stress marks on body parts; 3, stress marks with hunching. Movement reluctance of the mice was scored as 0, normal activity; 1, movement on the placement of near object; 2, movement only after lifting mice up; 3, no movement even on handling (Atiq et al. 2019).

2.6.4 Evaluation of food consumption and survival analysis

Consumption of food on each day was recorded to assess food intake pattern of the mice in different groups (n = 5). Anorexia is a common symptom of chemotherapy induced mucositis (Basile et al. 2019). Therefore, daily food intake demonstrated development of FU-induced mucositis and the effect of therapy. Moreover, survival analysis was performed by observing the death rate of mice in each group daily.

2.6.5 Effect of 5-FU induced mucositis on organs

At the end of experiment, mice were euthanized, and entire small intestine and colon were excised. The weight of small intestine and colon length were recorded after removal of feces and adjacent fats. The tissues were fixed in 10% formalin and frozen at -80°C for further analysis. The weight of spleen was evaluated as an inflammation index. In addition, liver and kidney were resected and weigh to evaluate the effect of chemotherapy and the treatment on these vital organs.

2.6.6 Histopathological evaluation

For histological analysis, a 1-cm excised tissue sample of small intestine (jejunum) and large intestine from each mice group was fixed in 10% buffered formalin and embedded in a paraffin wax. Afterwards, thin section of 5 µm thickness were incised from each sample, mounted on a glass slides and stained with hematoxylin and eosin (H & E). The prepared samples were observed microscopically at different magnifications (4x, 10x and 40x) to assess severity of intestinal damage. The histo-morphometric parameters for small intestine include measurement of small intestinal villus height (top of villus to villus-crypt junction; n = 20 villi), crypt depth (adjacent villi intussusceptions; n = 20) and calculating the index to estimate villi-crypt damage using TCapture imaging software (Tucsen Photonics Co. Ltd.) and a light microscope equipped with high resolution camera (ISH 500, Tucsen CMOS USB 2.0). The villi-crypts measurements were performed using ImageJ software (NIH, USA). While, large intestine histological score based on inflammation indexes including sum scores of surface epithelial loss, crypt destruction and inflammatory cells infiltration according to pre-determined scale (Erben et al. 2014).

2.6.7 Goblet cells evaluation

Similarly, 1 cm thin sections from each harvested small intestine (jejunum) were fixed in 10% formalin and embedded in paraffin wax. Then sections of 5 µm thickness were stained with periodic acid schiff-acian blue (PAS-acian blue) stain to visualize goblet cells and mucin content in the jejunum samples of each group (Ali et al. 2019; Stringer et al. 2009). The stained images were captured as mentioned above and processed to calculate number of intact goblet cells per villus, mucin-stained area containing MUC-2 protein and fluorescence intensity of the blue colored stain using ImageJ software. Results are expressed as mean ± SD.

2.6.8 Enzyme linked immunosorbent assay (ELISA) for cytokines profiling

The severity of intestinal inflammation was determined through expression of pro-inflammatory cytokines (IL-1 β , IL-6 and TNF- α) in the jejunum sections excised from each mice group (n = 5 mice/group) using ELISA kits (eBioscience, Inc., San Diego, CA, USA).

All assays were conducted according to the manufacturer's instructions.

2.6.9 Biochemical assays to determine intestinal antioxidant protection level

To assess antioxidant enzymatic activity in the intestine, 1 cm jejunum tissue was excised at the end of experiment from all groups (n = 5). The tissue was immediately immersed in phosphate buffer saline (PBS) (1 mL) and homogenized at 10,000 rpm until single cell suspension was obtained, then centrifuged (489g x 10 min) to get clear supernatant sample. Further, biochemical assays were performed to estimate antioxidant enzymatic levels of GSH, GST and catalase.

Reduced Glutathione (GSH) levels were determined in each mice group using Ellman's reagent method (Arruda et al. 2013; Moron et al. 1979) under UV/Vis spectrophotometer at 412 nm. PBS was used as a blank. The obtained GSH values were expressed as nmol/g tissue sample \pm SD.

Afterwards, Glutathione sulfotransferase (GST) assay was conducted to determine intestinal tissue detoxification level against oxidative damage. It was determined spectroscopically at 340 nm using potassium phosphate buffer as a blank. Assay mixture without supernatant was used as a control to determine non-specific binding of the substrates (GSH and 1-chloro-2,4-dinitrobenzene (CDNB)) (Arruda et al. 2013; Habig et al. 1974). GST activity was expressed as nmol of CDNB conjugated/g tissue weight.

Furthermore, catalase activity was determined to find its protective effects by mixing 3 ml of H₂O₂-potassium phosphate buffer (0.6 M) and 40ul of supernatant. Then decrease in absorbance was recorded spectrophotometrically at 240 nm. The same assay mixture without H₂O₂ was used as a blank. Results were stated as enzyme units/min/mg. One enzymatic unit is the time required to reduce the absorbance by 0.05 units at 240 nm (Hadwan and Abed 2016).

2.7 Statistical Analysis

Statistical analysis between the groups was conducted using one-way ANOVA or t-test. The significance level was considered 0.05* ($\alpha = 0.05$), 0.01** ($\alpha = 0.01$) and 0.001*** ($\alpha = 0.001$).

3. Results

3.1 Physicochemical characterization of the prepared GA-PLGA nanoparticles

GA-PLGA nanoparticles were successfully prepared with an average particle size of 190.46 \pm 7.49 nm, zeta potential of -11.67 \pm 2.5mV and PDI lesser than 0.3 (0.123 \pm 0.112). Particle size below 200 nm can

easily accumulate and reside at the targeted site (Collnot et al. 2012; Zeeshan et al. 2019a). Moreover, nanoparticles were fabricated with good percentage yield ($78 \pm 2.51\%$) and drug entrapment ($67.66 \pm 2.5\%$) (Table 1).

Table 1
Physicochemical characteristics of GA-PLGA nanoparticles

Formulations	Particle size (nm)	PDI	% Encapsulation efficiency	Zeta potential (mV)	% Yield
Blank PLGA NPs	157.6 ± 7.9	0.229 ± 0.15	N/A	-9.33 ± 3.21	75.33 ± 2.51
GA-PLGA NPs	190.46 ± 7.49	0.123 ± 0.112	67.66 ± 2.5	-11.67 ± 2.5	78.0 ± 2.64

*PDI-poly dispersity index

3.2 FTIR investigations

FTIR spectrum analyzed the functional groups within chemical structures of Glycyrrhizic acid, PLGA polymer and GA-PLGA nanoparticles to ascertain interactions, compatibility and drug entrapment (Fig. 2a-c). FTIR spectrum of Glycyrrhizic acid showed broad peak of OH at 3391 cm^{-1} , sharp peaks of C-CH₂ at 2926 cm^{-1} and 2855 cm^{-1} , C = O at 1745 cm^{-1} and 1634 cm^{-1} , CH₃ at 1458 cm^{-1} , OH in-plane bend at 1279 cm^{-1} , acetates at 1209 cm^{-1} , esteric C-O stretch at 1163 cm^{-1} , characteristic secondary cyclic alcohols C-O stretch at 1032 cm^{-1} , C-C peak at 979 cm^{-1} , C-C at 700 cm^{-1} , and NH out of plane at 615 cm^{-1} . Similarly, PLGA FTIR spectrum (Fig. 2) has depicted functional groups of free OH at 3517 cm^{-1} with lower intensity, peaks of C-CH₂ at 2926 cm^{-1} and 2855 cm^{-1} , C = O at 1746 cm^{-1} and 1629 cm^{-1} , other stretches of CH₃ at 1456 cm^{-1} , C-OH in-plane bend at 1427 cm^{-1} , CH₃ at 1381 cm^{-1} , esteric C-O stretch at 1163 cm^{-1} , primary alcohol C-O stretch at 1088 cm^{-1} and CH₂ at 719 cm^{-1} . Furthermore, GA-PLGA nanoparticles FTIR spectra (Fig. 2c) showed OH broadened peak at 3383 cm^{-1} , secondary cyclic alcohols C-O stretch at 1037 cm^{-1} and OH in-plane bend at 1276 cm^{-1} which characterized inside drug entrapment. Other representative peaks of both drug and PLGA are C-CH₂ at 2928 cm^{-1} and 2856 cm^{-1} , C = O at 1745 cm^{-1} and 1643 cm^{-1} , CH₃ at 1453 cm^{-1} and esteric C-O at 1161 cm^{-1} . Other visualized peaks in the spectra are for C-C vibrations at 980 cm^{-1} , C-C at 702 cm^{-1} and NH out of plane at 617 cm^{-1} . The fingerprint regions of both drug and polymer were retained in the spectra which confirmed compatibility and exclude possibility of chemical interaction or bond formation. A relative change in the intensity of transmittance showed an overlap of the same functional group of both drug and the polymer which confirmed drug entrapment (Fig. 2c).

3.3 Morphological analysis

SEM analysis confirmed the particle size within required size range ($< 200 \text{ nm}$). Further, SEM revealed nanoparticles morphological features as spherical in shape with smooth surface (Fig. 3).

3.4 In vitro drug release study

Drug release from the GA-PLGA nanoparticles was conducted in SGF (pH 1.2) for the first 2 hours, afterwards conducted in PBS (pH 6.8) at 37°C up to 48 hours. The pattern obtained for the drug release at pre-determined time points indicated initial burst release, followed by sustained release pattern up till 48 hours, accounting 77.67 ± 1.1547 % of drug release (Fig. 3). The drug release behavior is the typical pattern of drug release from PLGA nanoparticles (Ali et al. 2016).

3.5 Assessment of 5-FU mucositis induction and treatment through morphological and behavioral parameters

5-FU induction declines mice body weight, as manifested from daily weight assessment. It is one of the symptoms associated with intestinal inflammation. Treatment either with Glycyrrhizic acid (drug) or GA-PLGA nanoparticles restore mice body weight (Fig. 4a). However, the highest recovery in % body weight was noticed with GA-PLGA nanoparticles administration ($p < 0.001$). The two treatment groups differ by the factor 4, in terms of %BW (Fig. 4a). Furthermore, mice from all groups were assessed for stool consistency; initially softened stools were observed in all groups. After 3rd day, mice injected with 5-FU have suffered from pronounced diarrhea. Treatment groups relieved diarrheal symptoms to much extent, although not completely; GA-PLGA nanoparticles have shown significant improvement ($p < 0.001$) (Fig. 4b).

Induction of mucositis by 5-FU causes mice distress, as manifested from behavioral signs like difficulty to move, anxious behavior and fur condition (score = 3; $p < 0.001^{###}$), when compared with normal healthy mice. Glycyrrhizic acid and GA-PLGA nanoparticles showed significant stress reduction in the 5-FU induced inflamed mice. At the end of experiment, Glycyrrhizic acid have decreased stress score up to 1.67 ± 0.57 ($p < 0.05$) and GA-PLGA reduced stress up to 1.25 ± 0.5 points ($p < 0.001$), when compared to 5-FU group (Fig. 4c).

3.6 Evaluation of daily food consumption and survival analysis

Mortality rate was assessed in all groups. Percentage survival rate was lowest for the 5-FU group; it differs widely from the normal mice group. While, free Glycyrrhizic drug improved survival rate up to 40%, when compare with 5-FU induced group. Whereas, GA-PLGA nanoparticles have greater survival rate (60%), in comparison to both 5-FU and Glycyrrhizic acid treated mice (Fig. 4d).

Anorexia is most commonly linked with chemotherapy (Basile et al. 2019); therefore, food intake was assessed in the mice of all four groups on daily basis. After 4th day of 5-FU intraperitoneal injection, a sharp decline in food consumption was observed ($p < 0.001$), as compared to the normal healthy mice. At the end of experiment, anorexia aggravates, and food intake left up to 31% of the initial food intake in the 5-FU group. Treatment groups (Glycyrrhizic acid or GA-PLGA nanoparticles) eradicate anorexia to a great extent ($p < 0.001$) in the 5-FU induced mice. Improvement in the nutritional intake was about 59% of the

initial food intake in the mice treated with Glycyrrhizic acid, while it was up to 78% in the GA-PLGA nanoparticles administered mice (Fig. 5a).

3.7 Effect of 5-FU induced mucositis and GA-PLGA nanoparticles treatment on the organs

Effect of 5-FU on major organs like spleen, liver, kidney, small intestine and large intestine (colon) were investigated and compared with the treatment groups (Song et al. 2013). 5-FU significantly decreased weight of spleen ($p < 0.01$) and kidney ($p < 0.05$), whereas did not affect liver profoundly (Fig. 5b). Likewise, shortening of small intestine ($p < 0.05$) and colon ($p < 0.001$) was observed to a significant extent (Fig. 5c, d). Treatment groups, either Glycyrrhizic acid or GA-PLGA nanoparticles, retard the negative effects of 5-FU on organs, meanwhile improves splenic weight significantly ($p < 0.01$). Moreover, Glycyrrhizic acid restored kidney weight ($p < 0.05$) and GA-PLGA nanoparticles significantly normalizes the kidney weight ($p < 0.01$). Further, Glycyrrhizic acid recovered the length of small intestine and colon to a little extent, whereas, GA-PLGA nanoparticles have aptly lengthen the 5-FU afflicted small intestine ($p < 0.05$) and colon ($p < 0.001$) (Fig. 5c, d).

3.8 Histopathological evaluation

Histological microscopy revealed intact epithelial integrity, well oriented villi, and aligned crypt cells, lack of inflammatory infiltrates in normal small intestine (jejunum) and large intestine (colon). Whereas, 5-FU mediate marked injury to both small and large intestine, but more severely affect small intestine, as shown in Fig. 6a-e. 5-FU induced small intestine histomorphological damage is evident from disrupted epithelium, shortened and distorted villi, vacuolated crypt cells, necrosis and immune cells infiltrations. Marked decrease in villi length ($p < 0.001$) was noticed with slightly enlarged crypts ($p < 0.01$), which altered villi to crypt ratio ($p < 0.001$), as compared to normal intestine (Fig. 6a,c-e). Similarly, large intestine has pronounced deformation with epithelium loss, villi-crypt morphological destruction and immune cells intrusion (Fig. 6a,b). Treatment with Glycyrrhizic acid or GA-PLGA nanoparticles restored the histomorphological features to a wide extent. Small intestine villi-crypt length and ratio and large intestine histopathological scores were improved through Glycyrrhizic acid treatment of 5-FU mice. Likewise, GA-PLGA nanoparticles have repaired villi length ($p < 0.001$), crypt dept ($p < 0.01$) and villi to crypt ratio ($p < 0.001$) in the 5-FU induced mice to a significant level (Fig. 6a,c-e). In addition, large intestine histology was recovered through Glycyrrhizic acid ($p < 0.05$) and more substantial through GA-PLGA nanoparticles ($p < 0.001$) treatment, as compared to 5-FU led mucositis group (Fig. 6a,b).

3.9 Exploration of goblet cell count and mucin content

5-FU mediated inflammation resulted in the goblet cells decline and distortion in the jejunum, thus reducing the mucin content in the intestinal tissue. Treatment with Glycyrrhizic acid restore the goblet cell count to some extent ($p < 0.01$), while GA-PLGA nanoparticles increase the goblet cells number and architecture in the tissues predominantly ($p < 0.001$) (Fig. 7a,b). Furthermore, MUC-2 protein is mainly responsible for the mucin formation in the intestine (Tadesse et al. 2017), it was indirectly assessed through PAS-acian blue stained mucin area and resulting fluorescence intensity within goblet cells using

ImageJ software. Mucin stained area ($p < 0.01$) and fluorescence intensity ($p < 0.001$) was found to be significantly altered on 5-FU induction as compared to normal control (Fig. 7c,d). Glycyrrhizic acid have a limited increase in mucin overall area, however, enhanced fluorescence intensity profoundly. While, GA-PLGA nanoparticle markedly elevated the stained area ($p < 0.05$) and fluorescence ($p < 0.001$), as compared to 5-FU group (Fig. 7c,d).

3.10 Enzyme linked immunosorbent assay (ELISA) for cytokines profiling

5-FU induced mucositis manifested through predominant rise in the pro-inflammatory cytokines including TNF- α , IL-1 β and IL-6 (Chang et al. 2012; Song et al. 2013). To assess anti-inflammatory activity, pro-inflammatory cytokines were estimated in the excised jejunum tissues from all groups. Marked elevation in the pro-inflammatory cytokines level were noticed in the 5-FU induced mucositis group as compared to the normal healthy group. Treatment, either Glycyrrhizic acid or GA-PLGA nanoparticles, markedly decline TNF- α , IL-1 β and IL-6 levels $\geq 50\%$, thus exhibiting their activity against inflammation in the disease model (Fig. 8a-c).

3.11 Biochemical assays to determine intestinal protection level

5-FU associated intestinal inflammation generate excessive reactive oxygen species (ROS), whose overproduction resulted in diminished activity of antioxidant defensive enzymes (GSH, GST, catalase) (Chang et al. 2012; Gelen et al. 2018). In this study, 5-FU induced mice have shown considerable lowering in the antioxidant enzymes, GSH (3.487 ± 0.5 nmol/g; $p < 0.001$), GST (125 ± 9.13 nmol/g, $p < 0.001$) and catalase (0.316 ± 0.0352 U/min/mg, $p < 0.05$) in comparison to the normal mice GSH (81.2 ± 0.75 nmol/g), GST (845.2 ± 5.0 nmol/g) and catalase (0.527 ± 0.0775 U/min/mg) levels. Mice treated with plain Glycyrrhizic acid have elevated GSH and GST levels, however, did not affect catalase activity significantly. While, GA-PLGA nanoparticles have more pronounced effect in levelling-up of antioxidant protection in 5-FU induced mice, enhancing GSH, GST and catalase activity to 33.08 ± 1.42 nmol/g ($p < 0.001$), 600.33 ± 5.5 nmol/g ($p < 0.001$) and 0.4 ± 0.0168 U/min/mg ($p < 0.05$) respectively (Fig. 8d-f).

4. Discussion

5-FU, an anti-pyrimidine and antimetabolite drug, is widely used for chemotherapeutic purpose to treat malignant tumors of colorectal and breast regions (Chang et al. 2012; Chen et al. 2020). However, the continuous use of 5-FU is associated with intestinal mucositis leading to destruction of microstructure of intestinal like villi, crypts, epithelial cells and goblet cells and manifested through clinical symptoms like body weight loss, heavy diarrhea, anorexia and anxiety (Song et al. 2013). 5-FU led mucositis severely compromised patient's compliance receiving anti-neoplastic chemotherapy and cancer treatment must be discontinued due to severe mucositis.

Glycyrrhizic acid is a marvelous herbal medicine, used to cure several ailments from centuries. Recently, it was found effective in the treatment of methotrexate induced enteritis (Wang and Du 2016). Owing to its anti-inflammatory, anti-ulcer, mucoprotective, immunomodulatory, anti-bacterial, antioxidant and anti-cancerous actions (Ming and Yin 2013), it is the suitable candidate to protect intestine from injury. However, its protective effect is limited in the intestine because of rapid elimination from the gut in response to heavy diarrhea and degradation by indigenous enzymes. Therefore, a sustained therapeutic effect can be achieved by encapsulating Glycyrrhizic acid inside a bio-compatible nanocarrier. In our previous research, Glycyrrhizic acid loaded polymeric nanocarriers proved to be efficient in ameliorating DSS induced ulcerative colitis (Zeeshan et al. 2019b). However, the scientific evidence of Glycyrrhizic acid and its nano carrier is not available to prove its efficacy against 5-FU induced intestinal mucositis. PLGA is widely used biocompatible, biodegradable, and harmless polymeric carrier inside the living systems. Therefore, PLGA was used to prepare Glycyrrhizic acid loaded PLGA nanoparticles using double emulsion method in this study. Previously, it was found to be a sustained drug release carrier in the treatment of oral mucositis (Takeuchi et al. 2018). Thus, PLGA would be an ideal carrier to be explored for 5-FU led mucositis of the intestines.

The synthesized nanoparticles have optimal size range < 200 nm with lesser PDI, indicating monodispersed system. The drug has suitable encapsulation efficiency and circular morphology (Table 1, Fig. 3). Particles with size lesser than 200 nm accumulate in the inflamed tissues through eEPR effect and prone to rapidly uptake by the immune cells recruited as a consequence of inflammation (Zeeshan et al. 2019a). Further, invitro drug release studies indicated initial burst release of drug, followed by sustained release drug behavior till 48 hours (Fig. 3). It was found to be consistent with previous findings (Zeeshan et al. 2019b).

To explore, preventive and therapeutic role of GA-PLGA nanoparticles against chemotherapeutic induced intestinal injury, 5-FU induced intestinal mucositis in the murine mice was developed via intraperitoneal injection according to the mentioned protocol (Fig. 1) (Atiq et al. 2019). The features of experimental animal model are nearly the same as found in the reported studies (Gelen et al. 2018; Zhang et al. 2017), explicated through diarrhea, loss in body weight, lower food intake, histological architecture destruction (villi and crypts lengths), mucin depletion and decreased goblet cell count. In addition to 5-FU IP injection, treatment groups received once daily intake of Glycyrrhizic acid free drug or GA-PLGA nanoparticles through oral route. Oral route is most preferable in this condition since it completely covers the natural way to intestines. Both, Glycyrrhizic acid and GA-PLGA nanoparticles alleviates the clinical symptoms and reduced the severity of diarrhea, weight loss, anorexia and distress level (Fig. 4, Fig. 5). Aggressive behavior was very prominent in the untreated mucositis mice. Similarly, the 5-FU induced mucositis highly increased the mortality rate, which was slow down by the Glycyrrhizic acid or GA-PLGA nanoparticles treatment (Fig. 4). Although free drug proved to be effective in remitting the symptoms and increases the survival chance, however, GA-PLGA nanoparticle always turned out to be more efficacious which paved a way for PLGA and other polymeric nanocarrier systems to be further explored in the management of intestinal mucositis.

In previous animal experimental models, 5-FU significantly affect weight of spleen, kidney and to lesser extent liver (Gelen et al. 2018; Whittaker et al. 2016; Yang et al. 2017). In the current experiment, visceral organs weight assessment indicated that 5-FU decline splenic weight about 66% and kidney weight about 32%, as compared to the normal control (Fig. 5). Although, 5-FU did not significantly alter liver weight, but accounting for 22% decline. The weight decline possibly driven because of 5-FU induced excessive inflammatory response or immunosuppressive action of 5-FU that decreases splenic weight (Whittaker et al. 2016). Further, 5-FU shorten length of small intestine and large intestine (colon) (Fig. 5). Glycyrrhizic acid plain drug restore colon to an appropriate length ($p < 0.01$), but lesser lengthening effect on small intestine (Fig. 5). Whereas, GA-PLGA nanoparticles endure greater protection as manifested from significant increase in both small intestine ($p < 0.05$) and colonic length ($p < 0.001$).

Moreover, intestinal mucosal and epithelial layer destruction by 5-FU were efficiently restored through Glycyrrhizic acid or GA-PLGA nanoparticles administration. Our findings indicated that 5-FU induced histomorphological damage as manifested through shortened villi and crypt lengths, disintegrated epithelium, immune cells intrusion and goblet cells loss has been alleviated through Glycyrrhizic acid or GA-PLGA nanoparticles treatment. Constantly, GA-PLGA nanoparticles have superior mucoprotective effects as compared to free drug alone (Fig. 6). The results suggest that recovery of intestinal epithelia and mucosal damage could be the possible reason behind reduction in diarrhea (Zhang et al. 2019). Additionally, the histopathological findings were supported through investigating goblet cells count and mucin content. Consistent with previous data, 5-FU distort barrier function through loss of goblet cells number and disintegration of its structure leading to mucin discharge and depletion (Ali et al. 2019; Stringer et al. 2009). Again, the treatment elevated goblet cell count and MUC-2 protein containing mucin content in the intestine, with more notable improvement in GA-PLGA nanoparticles group (Fig. 7).

To date, pathogenesis of 5-FU mediated intestinal mucositis is not completely elucidated. However, several underlying elements that are responsible for mucositis damages DNA and cellular and sub-cellular structures because of overproduction of ROS, uncontrolled inflammation that destroy intestinal homeostasis and natural microbial flora alterations (Yan et al. 2020). In fact, ROS excessive production trigger the inflammatory pathways and pro-inflammatory cytokines production (Gelen et al. 2018; Yan et al. 2020). Among many others, some basic transcription factors activated by ROS are NF- κ B and MAPK proteins that increase the production of pro-inflammatory cytokines including TNF- α , IL-6 and IL-1 β (Chang et al. 2012; Song et al. 2013; Zhang et al. 2017). Elevation of pro-inflammatory cytokines activates a series of acute reactions to damage cells and tissues, thus causing mucosal injury and mucositis. Interestingly, Glycyrrhizic acid has been explored to mediate its action through inhibition of NF- κ B and MAPK pathways (Wang and Du 2016), thus reducing pro-inflammatory cytokines production and resulting inflammation. In this study, Glycyrrhizic acid as a free drug or encapsulated within nanoparticles reduced the expression of TNF- α , IL-6 and IL-1 β cytokines, thus protecting intestinal mucosa from 5-FU induced damage. More significant reduction was found to be with the encapsulated drug within nanoparticles (Fig. 8).

Furthermore, 5-FU led inflammation and ROS overproduction has weakened body's defensive antioxidant system (Yan et al. 2020). Thus, the antioxidant protective enzymes could not be able to capture excessive ROS, leading to increased oxidative stress and cellular damage. In the present study, 5-FU depleted antioxidant enzymes including GSH, GST and catalase (Fig. 8) and causing mucosal damage. Glycyrrhizic acid proved to be an anti-inflammatory and antioxidant drug (Ming and Yin 2013); and reduces oxidative stress in DSS-induced colitis through elevation of antioxidant protective enzymes (Zeeshan et al. 2019b). Consistent with the previous findings, both free drug and encapsulated drug increased mucosal protection through rise in GSH, GST and catalase levels. While, GA-PLGA nanoparticles exhibited more pronounced effect (Fig. 8), because of sustained drug release at the targeted intestinal tissues with more localized therapeutic effect.

Hence, Glycyrrhizic acid proved to be an ideal candidate in alleviating intestinal mucositis, whereby, its pharmacokinetics limitation and prolonged protective and therapeutic effect is achieved through encapsulating it in PLGA polymeric nanocarrier. PLGA nanoparticles with their inert nature successfully deliver the drug to the inflamed intestinal tissues and enhanced therapeutic action through accumulation of nanoparticles at the site and minimizing drug clearance due to diarrhea.

5. Conclusion

The present work concluded that GA-PLGA nanoparticles with appropriate physicochemical properties and drug release behavior could be a promising approach to protect and cure small and large intestine against 5-FU induced intestinal mucositis. GA-PLGA nanoparticles effectively reverse the 5-FU led damage by improving clinical symptoms, behavioral manifestations, histomorphological architecture and regulation of intestinal homeostasis through minimizing oxidative stress and pro-inflammatory cytokines. Together, GA-PLGA nanoparticles could serve as a protective and therapeutic option to alleviate 5-FU induced mucositis and further investigated into clinics for large scale benefits.

Declarations

Funding:

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Ethics approval:

All animal studies were approved and performed according to the bioethical committee protocols of Quaid-i-Azam University, Islamabad for the care and use of lab animals (Approval no. BES-fbs-QAU2018-75)

Disclosure statement:

Authors declared no conflict of interests.

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Figures

Figure 1

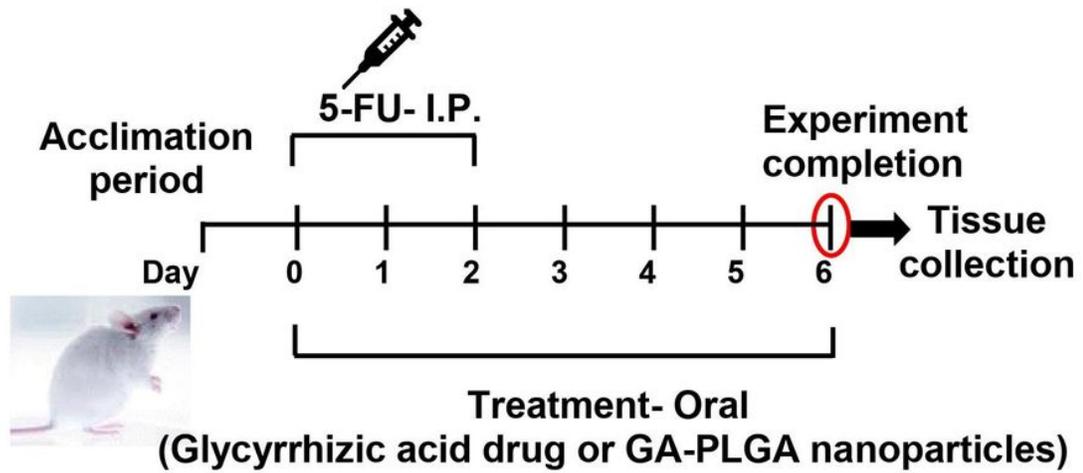


Figure 1

Experimental design for induction of intestinal mucositis through 5-FU intraperitoneal injection (50mg/kg) and treatment with oral administration of Glycyrrhizic acid plain drug or Glycyrrhizic acid loaded PLGA (GA-PLGA) nanoparticles (10mg/kg)

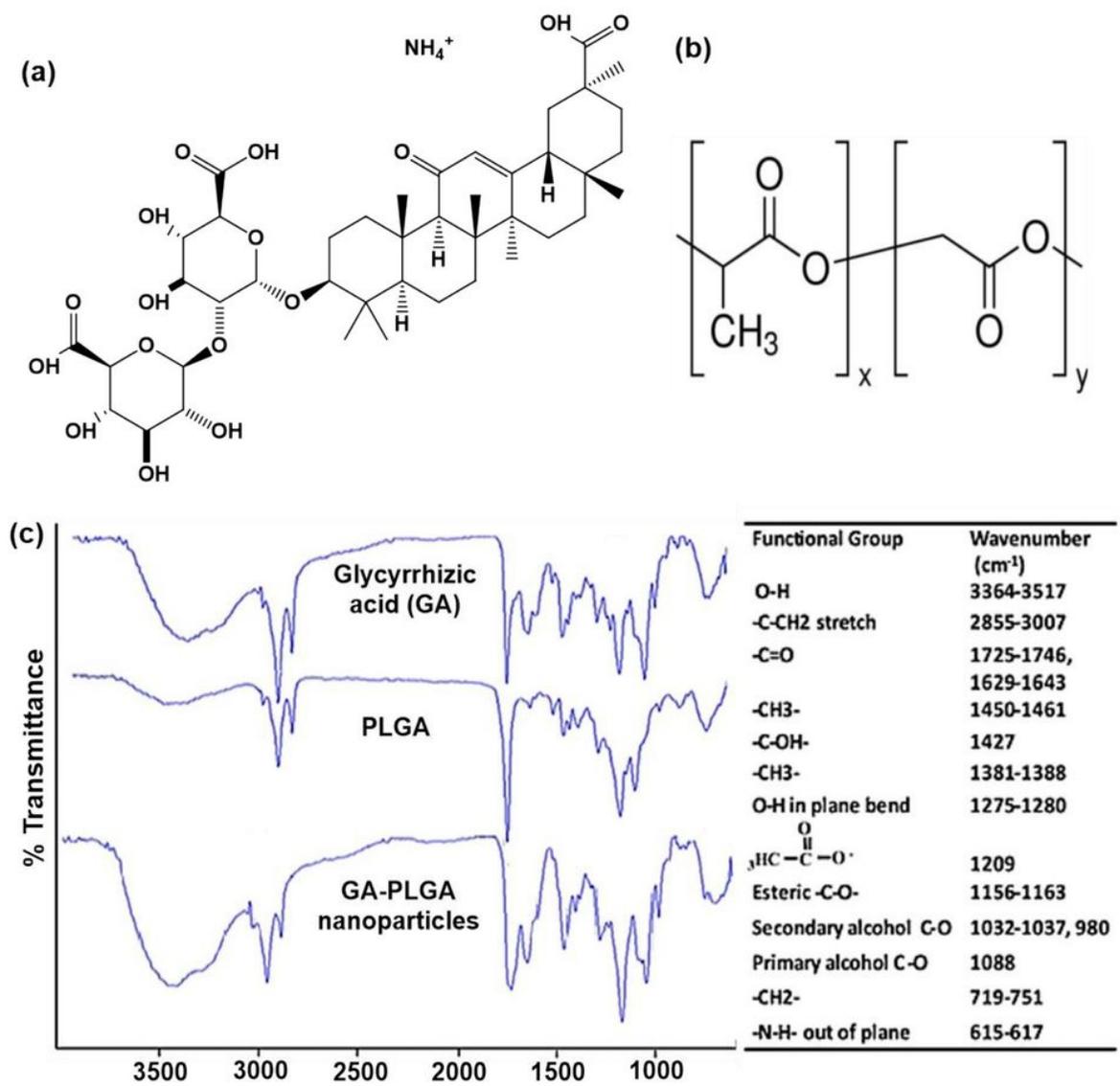


Figure 2

(a) Chemical structure of Glycyrrhizic acid (monoammonium salt) (b) Chemical structure of PLGA (c) FTIR spectra of Glycyrrhizic acid, PLGA polymer and GA-PLGA nanoparticles

Figure 3

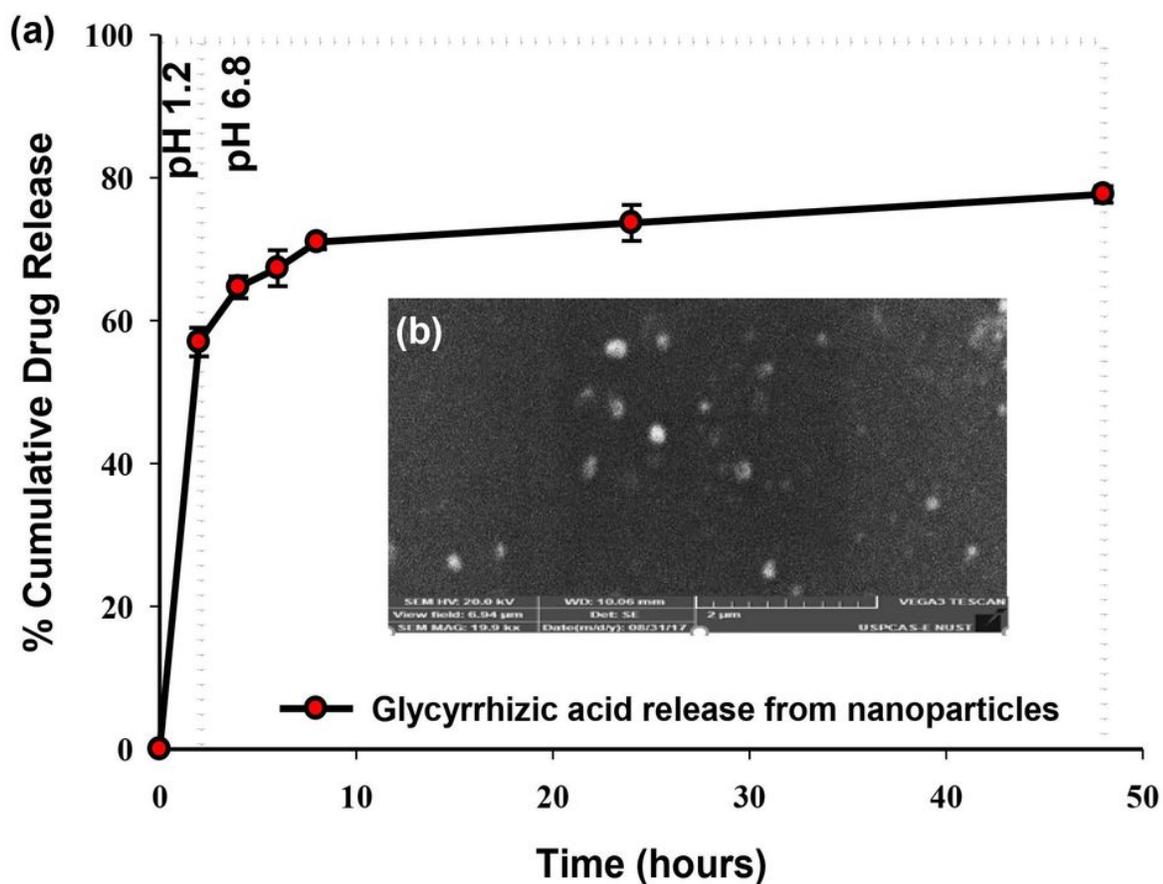


Figure 3

(a) Cumulative drug release from synthesized nanoparticles (n=3) (b) Scanning electron microscopic (SEM) image of GA-PLGA NPs

Figure 4

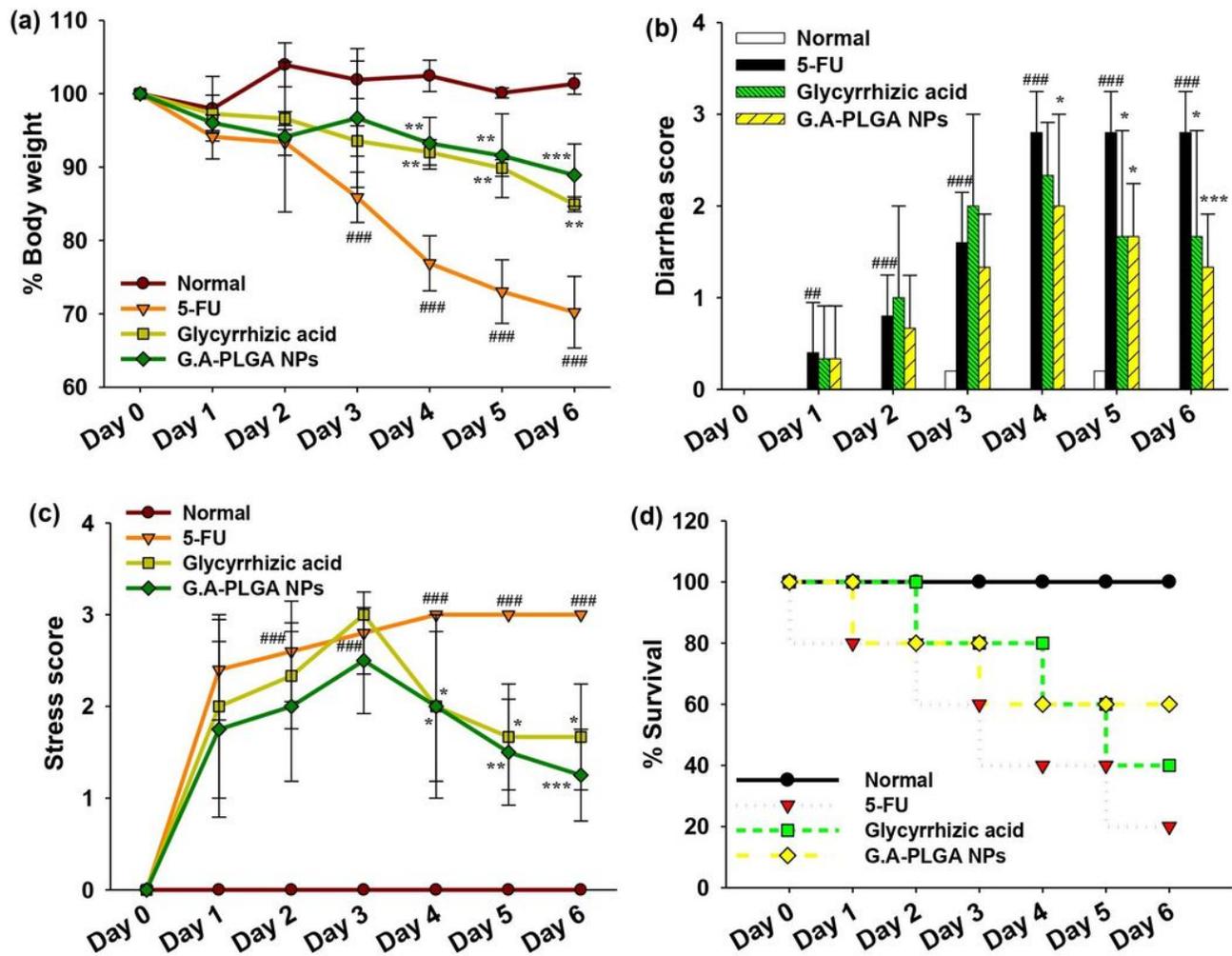


Figure 4

(a) Assessment of % daily body weight loss (b) stool consistency (c) stress (d) % mice survival in normal healthy control, 5-FU induced mucositis, Glycyrrhizic acid treated and GA-PLGA nanoparticles treated groups (n=5/ group). Statistical significance from normal control group p<0.05#, p<0.01##, p<0.001###; and from 5-FU group p<0.05*, p<0.01**, p<0.001***

Figure 5

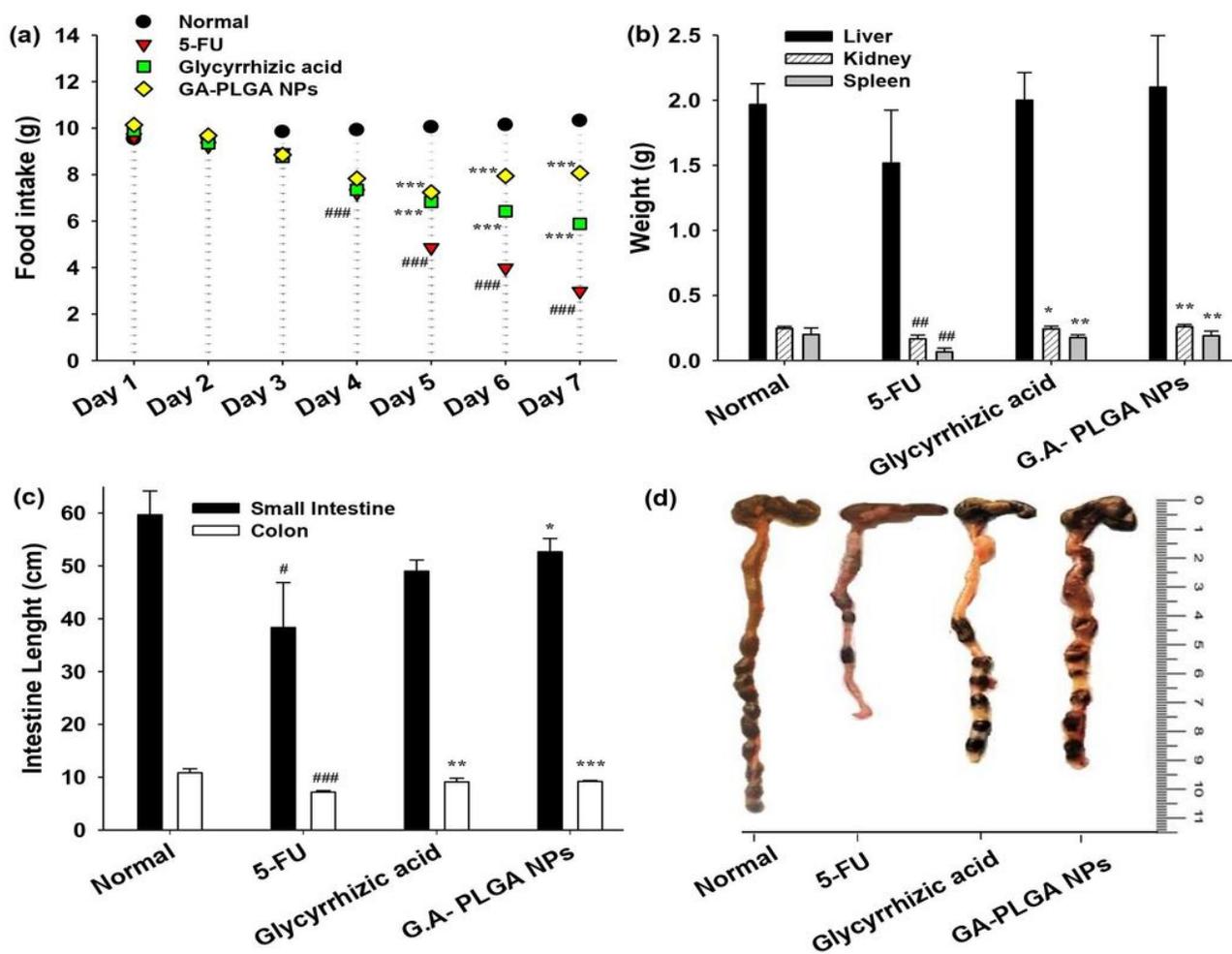


Figure 5

(a) Estimation of daily food intake of the mice in all groups (n=5/ group) (b) Effect of 5-FU induction and treatment groups on weight of major organs (c, d) and length of large intestine (colon). Statistical significance from normal control group $p < 0.05$ #, $p < 0.01$ ##, $p < 0.001$ ###; and from 5-FU group $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***

Figure 6

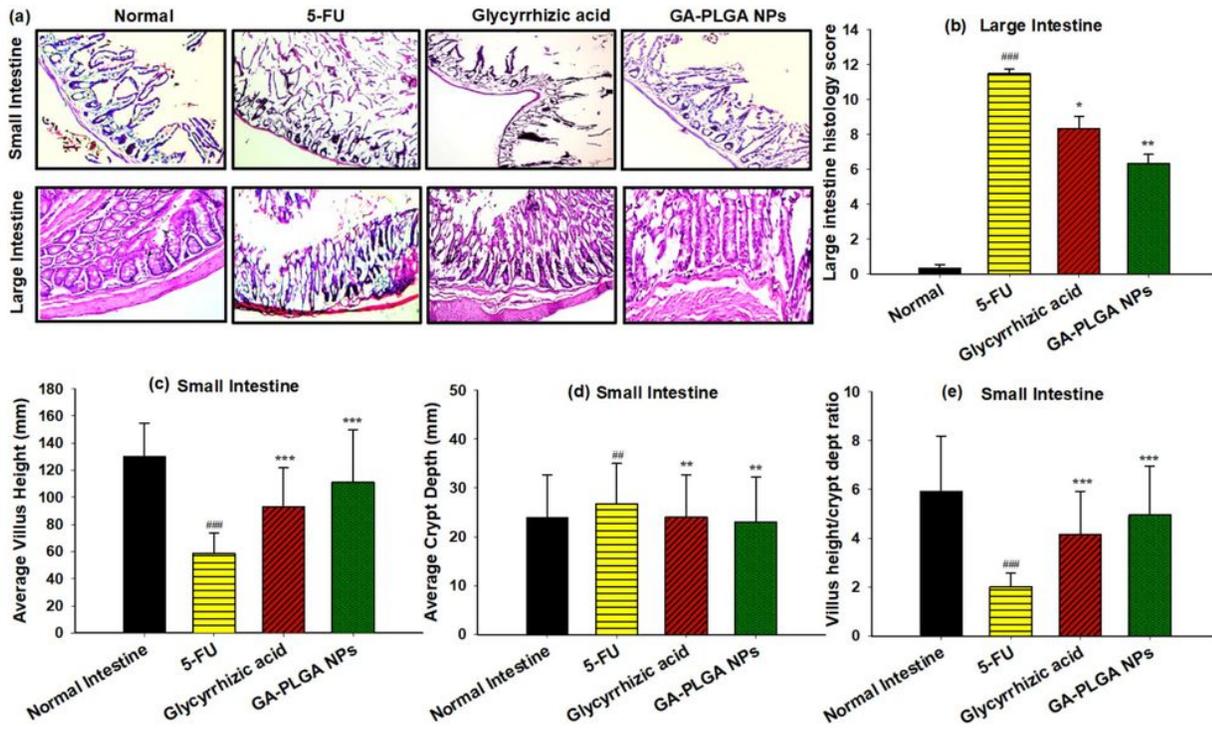


Figure 6

(a) Histopathology of the excised small and large intestines from normal mice, 5-FU induced mucositis group, Glycyrrhizic acid and GA-PLGA nanoparticles treated groups (b) Histological scores of large intestine on a scale of 0-12 And histo-morphometric evaluation of small intestine (jejunum) including (c) villi height (μm), (d) crypt dept (μm) and (e) villi height/crypt depth ratio found for each group (n=5). Statistical significance from normal control group $p < 0.05$ #, $p < 0.01$ ##, $p < 0.001$ ###; and from 5-FU group $p < 0.05$ *, $p < 0.01$ ** , $p < 0.001$ ***

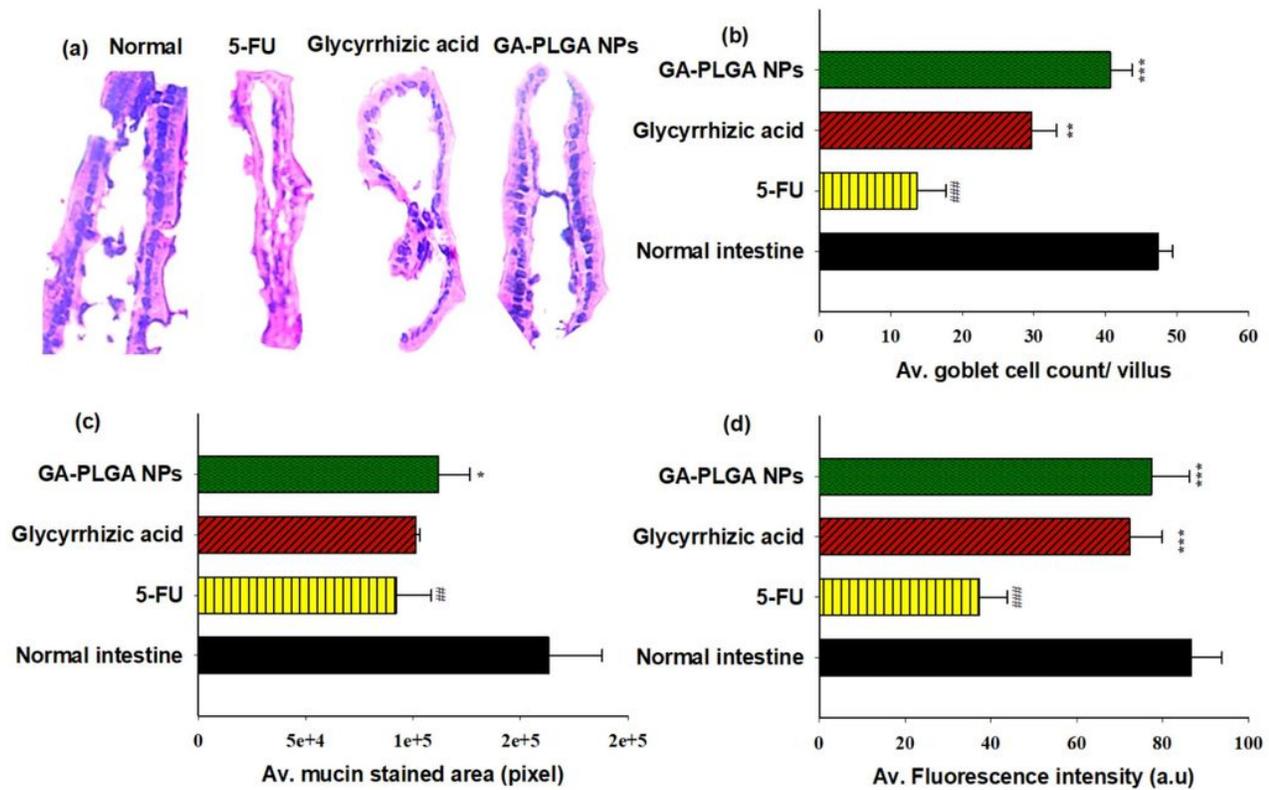


Figure 7

(a) PAS-acian blue staining of jejunum excised from normal control, 5-FU induced mucositis group, Glycyrrhizic acid and GA-PLGA nanoparticles treated groups (b) Average number of goblet cells per villus in each group (n =20 villi taken from three different samples) (c) Average mucin-stained area (pixels) in each group (d) Average fluorescence intensity of blue mucin stain in the jejunum of each group. Statistical significance from normal control group $p < 0.05$ #, $p < 0.01$ ##, $p < 0.001$ ###; and from 5-FU group $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***

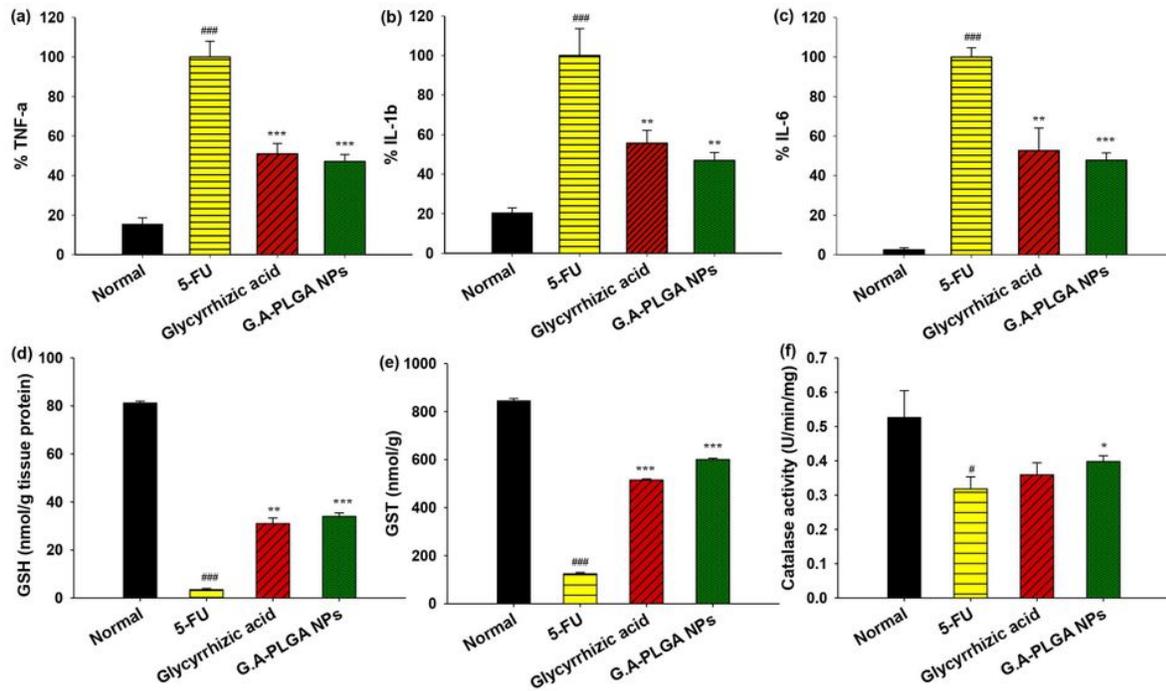


Figure 8

Relative expression of pro-inflammatory cytokines, (a) TNF- α , (b) IL-1 β and (c) IL-6 in the normal control, 5-FU induced mucositis group, Glycyrrhizic acid and GA-PLGA nanoparticles treated groups (n=5/ group). Evaluation of antioxidant protection of the treatment against 5-FU through estimation of (d) GSH (e) GST and (f) catalase enzyme in the jejunum tissue excised from each group (n=5/ group). Statistical significance from normal control group p<0.05#, p<0.01##, p<0.001###; and from 5-FU group p<0.05*, p<0.01**, p<0.001***

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