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Mucoprotective effects of Saikosaponin-A in 5-fluorouracil-induced intestinal mucositis in mice model

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ABSTRACT

5-Fluorouracil (5-FU)-induced intestinal mucositis (IM) is one of the most common oncological problem. It involves serious clinical consequences such as diarrhea, erythematous lesions of mucosa, and eventually development of ulcers accompanied by severe pain. The aim of the present study was to demonstrate the mucoprotective effects of Saikosaponin-A in 5-FU-induced intestinal mucositis in mice. Mucositis was induced in BALB/c mice by intraperitoneal injection of 5-FU (50 mg/kg/day) for three consecutive days and IM was assessed by both behavioral and histochemical analysis. While, Saikosaponin-A (1, 5, 10 mg/kg/day) was administered 1 h before 5-FU injection for consecutive seven days. Pre-treatment of Saikosaponin-A significantly ameliorated the severity of mucositis reflected as food intake, body weight loss, severity of diarrhea and mortality rate in a dose depended manner as compared to mice treated with 5-FU. Moreover, histopathological analysis furthered reinforced the mucoprotective potential of Saikosaponin-A against 5-FU-induced intestinal abnormalities referred as villus atrophy, mitotic crypt stem cells damage, inflammatory cells infiltration, vacuolization and edema. Furthermore, Saikosaponin-A administration strongly inhibited pro-inflammatory mediators (TNF-α, COX-2, IL-1β and IL-6) and apoptotic markers (p-JNK, Casp-3). Saikosaponin-A pre-treatment significantly reduced the production of nitric oxide (NO) in intestinal tissue, inhibited acetic acid-induced Evans blue vascular permeability. The Siaikosaponin-A treatment markedly enhanced the anti-oxidants enzymes (Nrf2, HO-1, SOD, GSH, GST and Catalase), while decreased the oxidative stress markers i.e. Malonaldehde (MDA). Hence, these data suggest that Saikosaponin-A maybe a potential candidate for the treatment of chemotherapy-induced intestinal mucositis.

1. Introduction

It is estimated that 50–80% of patients suffered from intestinal mucositis during course of chemotherapy followed by clinical manifestation of ulceration, diarrhea and abdominal pain [1]. The whole process of mucositis occurs specially in epithelia having high rates of proliferation and turnover [2]. During mucositis epithelial layer passes through different developmental stages of cellular infiltration followed by a period of apoptosis, restitution and finally re-epithelialization of gut [3]. The architecture of epithelium maintained due to continuous mitotic activity of stem cells in crypts which due to apoptosis results in shallowing of crypts [4].

Mucositis is a complication related to radiation and chemotherapy specifically damaging mucosal lining of gastrointestinal tract compromising of five basic stages named as initiation, primary damage response, signal amplification, ulceration and healing [5]. First stage occurs after administration of chemotherapy, which involves DNA strand breaks and simultaneous production of reactive oxygen species (ROS). While, ROS formation results in cellular injury, targeting basal epithelium along with submucosa [6]. Second stage involves DNA damage, non-DNA damage and ROS which initiates a series of signal transduction pathways that activate various transcriptional factors like NF-κB [7]. Third stage involves signal amplification by transcriptional factors, as a consequence of broad range of accumulation of pro-inflammatory cytokines, damaging tissue and providing positive-feedback for primary tissue damage by chemotherapy [8]. In fourth stage

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mucosal architecture lost followed by painful lesion, providing a gateway for microbial entry [9]. Final stage, a stage of epithelial-cell migration, proliferation and differentiation at ulcer site [3]. Cytotoxic drugs inhibit DNA synthesis primarily interfere with thymidylate synthesis and trigger apoptosis through production of reactive oxygen species [10]. At the same time transcription factor, nuclear factor kappa B (NF-κB) activation leads to up-regulate various pro-inflammatory genes especially pro-inflammatory cytokines, which exaggerate tissue damage [11].

Additionally, the intestinal epithelium is highly sensitive to various stresses such as radiation i.e. total body irradiation or whole abdominal irradiation [12]. The 8–15 Gy γ-irradiation dose is enough to induce the alteration of the intestinal integrity and cause injury in animals [12]. However, the intestinal epithelium membrane have tremendous potential of regeneration following exposure to the dose of less than 14 Gy, while the dose above 14 Gy can cause acute radiation syndrome and increase mortality [12]. The regeneration of the intestinal epithelium can be categorized into three phases such as apoptotic phase, proliferation and growth, and finally into the normalization process [12]. The apoptotic process continues for two days following challenge with the radiation, and this process was confirmed with the histological and immunostaining procedure and characterized by the crypt loss, crypt size shrinkage and villi shortening during the stated phase [12]. The second phase that is regenerative phase continues from 2-4 days and the entire crypts are regenerated from the surviving crypt cells [12,13]. However, the number of the crypt cells decrease in both small and large intestine generally. Because of the rapid proliferation of the crypts cells during the regenerative phase the crypts enlarge in their size two folds. During third phase i.e normalization, the length of the villi and the crypts size becomes normalized to the pre-irradiation state [12,13]. The various medications used to deal with the colitis and crohn's disease includes monoclonal anti-bodies, immunomodulators and salicylic acid derivatives such as mesalazine and sulphasalzine etc [13]. However, these medications have their own limitation in one or the other way, therefore, exploring new therapeutic avenues are utmost important [13].

The natural product are the key and chief source of new chemical entity and plants offer the most important source of natural products. Saikosaponin-A is a triterpene saponin derived from *Bupleurum falcatum*. L (Umbelliferae) having a variety of pharmacological activities including anti-inflammatory, antioxidant, anti-bacterial, immunomodulatory, anti-allergic, anti-tumor and also reduction in cyclooxygenase, lipoxygenase production *in-vitro* have been demonstrated [14–16]. Based on its anti-inflammatory and anti-oxidant activities the present study aimed to explore the mucoprotective activity of Saikosaponin-A in 5-FU-induced intestinal mucositis specially targeting pro-inflammatory cytokines mediated mucosal damage both *in-vivo* and *in-silico*.

2. Materials and methods

2.1. Materials

Saikosaponin-A (purity ≥98%), was obtained from Professor Yeong Shik Kim College of pharmacy, Seoul National university, Seoul South Korea), Mesalazine and 5-Fluorouracil purchased from Sigma-Aldrich (St. Louis, MO, U.S.A). Primary anti-bodies such as mouse anti-phosphorylated p-c-Jun N-terminal kinase (p-JNK), mouse anti-COX-2, mouse anti-Caspase 3 and mouse anti-TNF-α antibodies (Santa Cruz Biotechnology, Inc). Secondary ABC reagents used in immunohistochemistry obtained from (SCBT U.S. A). ELISA kits for cytokines analysis were purchased from eBioscience (Inc San Diego, CA, U.S.A), while L-Glutathione reduced was purchased from (Sigma Aldrich, USA).

2.2. Animals

One forty male BALB/c mice having age 5–6 weeks, weight ranging between 25-30 g were purchased from National Institute of Health, Islamabad (Pakistan). All animals were acclimatized in ventilated cages for one week with standard conditions of temperature 20–25 °C, relative humidity 40–60%, 12:12 h light: dark cycle and provided mice with feed and pure drinking water *ad libitum*. Animal handling and all experimental procedures were in accordance with the Animal Care Ethical committee of Quaid-i-Azam University Islamabad, Pakistan (Ethical Approval; number BEC-FBC-QAU2018-85).

2.3. Experimental design

To determine IM, mice were randomly separated into four groups of eight mice each. Group I (Vehicle control) received 0.9% normal saline by oral route, Group II (Negative control) injected intraperitoneally (i.p) with 5-FU (50 mg/kg), Group III (Positive control) injected intragastrical Mesalazine (10 mg/kg), while Group IV (Treatment control) animals were administered with three different doses (1, 5, 10 mg/kg, i.p) of Saikosaponin A. In order to assess the survival analysis animals were randomly assigned to six groups and each group comprises of the 10 animals. The animals were randomly assigned to various groups such as vehicle control, 5-FU-induced, mesalazine 10 mg/kg, and Saikosaponin-A in three different dose i.e 1, 5 and 10 mg/kg. However, for the acetic acid-induced Evans blue vascular permeability, the animals were divided into the 4 groups (n = 8) i.e. acetic acid-induced, mesalazine 10 mg/kg and two groups of saikospaonin-A 5 mg/kg and 10 mg/kg.

2.4. Experimental mucositis induction

All animals except vehicle control were given 5-FU (50 mg/kg, i.p) for consecutive 3 days according to previously described protocol with slight modifications [13]. Pre-treatment (1 h before) of both Mesalazine and Saikosaponin-A were given once daily up to 7 days. Animals were sacrificed on 7th day under deep anesthesia with chloroform. The whole experimental design was shown in the Fig. 1.

2.5. Physical manifestations

Animals were monitored for disease severity by measuring food consumption, water intake, weight loss, distress symptoms and artificial system scoring of stool consistency: 0, Normal (normal stool, diarrhea absent): 1, Mild (slight wet/mushy stool indicating a mild diarrhea): 2, Intermediate (unformed watery stool indicating intermediate diarrhea): 3, Severe (bloody watery stool, indicating severe diarrhea): with the help of this grading system we analyze IM severity from diarrhea of normal grade (0) to diarrhea of severe grade (3) [17].

2.6. Tissue collection

On day 7th animals were killed by a deep anesthesia of chloroform and blood was collected through cardiac puncture. Blood samples were centrifuged at 3000 g for 5 min. The plasma obtained after centrifugation was stored at −80 °C for biochemical analysis. Both small and large intestinal segments were dissected from all groups and their

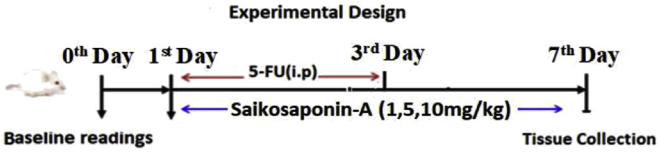

Fig. 1. Experimental design for mucositis induction by 5-FU administration.

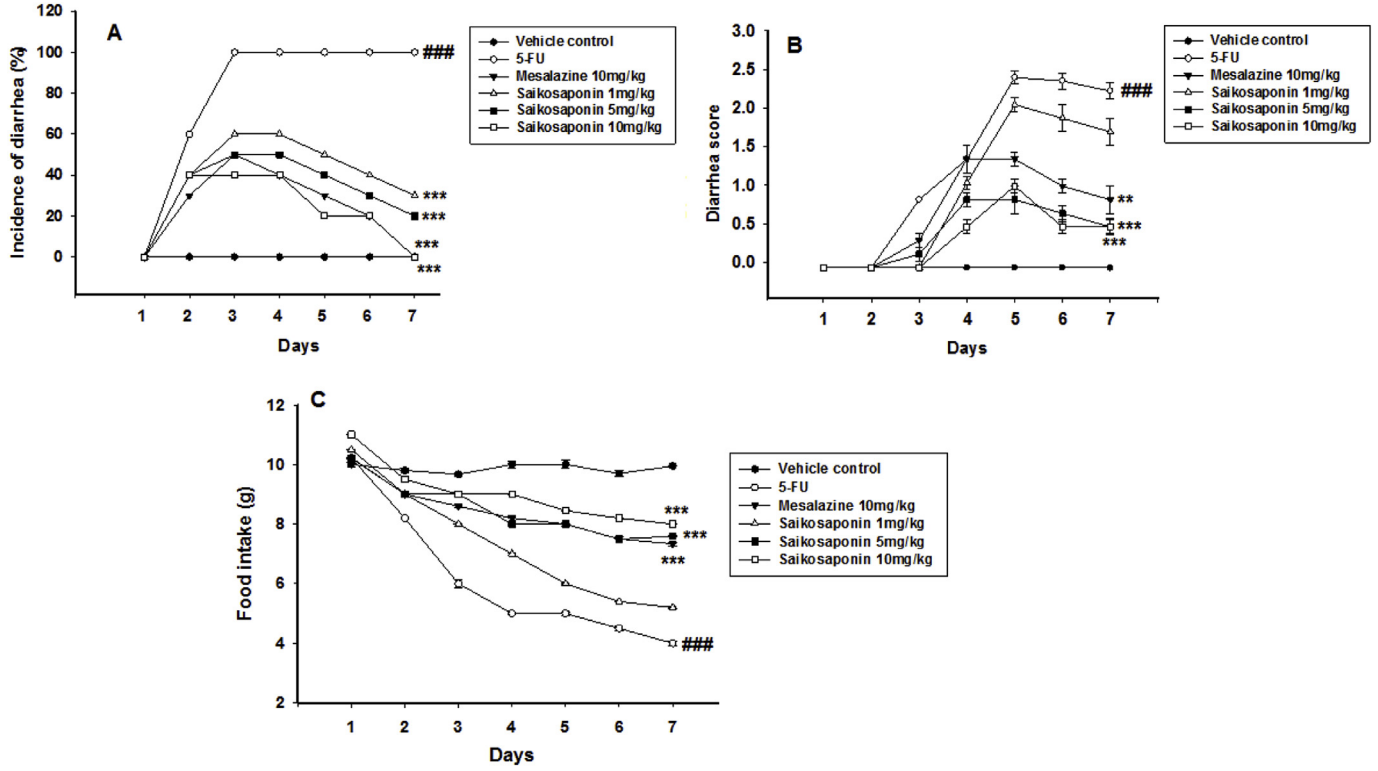

Fig. 2. Effect of Saikosaponin-A pretreatment on incidence of diarrhea (A), diarrhea score (B) and food intake (C). (A) Data showed percent incidence of diarrhea at different time intervals. Severity of diarrhea is evident from 72 h (Day-3) to 144 h (Day-6) after 5-FU administration. As compared to 5-FU group, Saikosaponin-A pretreatment reduce this severity from 120 h (Day-5) to mild and moderate type. Data represented the diarrhea score evaluation. All the mice were monitored during whole experiment for diarrhea score which was based on stool consistency. Artificial grading system for scoring of stool consistency has following criteria: 0, Normal (normal stool, diarrhea absent): 1, Mild (slight wet/mushy stool indicating a mild diarrhea): 2, Intermediate (unformed watery stool indicating intermediate diarrhea) 3, Severe (bloody watery stool, indicating severe diarrhea). Similarly, the food intake was also assessed in all treated groups. Data were expressed as the mean ± SD (n = 8 mice/groups). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ indicates significant difference of each treatment as compared to negative control group (only 5-FU). $^{\#\#\#}P < 0.001$ indicates significant difference of negative control group (only 5-FU) as compared to vehicle control group (only normal saline).

length were measured.

2.7. Histological examination and alcian blue-periodic acid schiff (PAS) stain

Segments of small and large intestine were collected for hematoxylin and eosin (H&E) staining. A segment of 3–5 cm was excised from all parts of intestine and rinsed with 0.9% normal saline. Segment of 2 cm from each group is fixed in 10% buffered neutral formalin for 24 h, dehydrated in ascending series of ethanol concentration and embedded in paraffin wax. Sections of 4 μm thickness were cut and fixed on glass slide for H & E stain. Histological assessment was performed under light microscope with calibrated micrometer at different magnification (4x, 10×, 40× Magnification) for measuring Villus heights (from villus tip to villus-crypt junction) Crypt depth (invagination depth between adjacent villi), crypt cell apoptosis and mitotic indexes. Twenty well-oriented villi and crypts (three samples per group) for villus height, crypt depth, mitotic figures per crypt were measured and scored for average of each sample. All intestinal morphometric assessment was performed blindly by ImageJ software 1.8_172 (NIH, USA).

In order to determine mucous secretion from goblet cells, sections from different groups were stained with Alcian Blue-PAS stain as described previously [18]. After dewaxing and rehydrating sections were stained in Alcian Blue (1% Alcian Blue 8GX (CI 74240) and (3% glacial acetic acid) for 5 min. After rinsing in distilled water, section was oxidized in 1% periodic acid and finally treated with Schiff reagent for 15 min. Total number of cavitated and goblet cells in at least 15 villi/section were calculated.

2.8. Immunohistochemical staining

Immunohistochemical staining was performed as previously described method with minor modification [19]. The slides were processed for the antigen retrieval step (enzymatic method) then, washed with PBS. The endogenous peroxidase was quenched by applying 3% hydrogen peroxide (H_2O_2) in methanol for 10 min. The slides were incubated with 5% normal goat serum containing 0.1% Triton X-100. After being blocked, the slides were incubated overnight in mouse anti-phosphorylated p-c-Jun N-terminal kinase (p-JNK), mouse anti-COX-2, mouse anti-TNF-α, mouse anti-Caspase 3 antibodies, mouse anti-Nuclear factor erythroid 2-related factor 2 (Nrf2) and mouse anti-hemoxygenase-1 (HO-1). At the early morning, after being washed with 0.1 M PBS, the slides were treated with biotinylated secondary antibodies according to origin of the primary antibodies and serum used, then successively with ABC reagents for 2 h in a humidified chamber. The slides were washed with 0.1 M PBS, stained in DAB solution, washed with distilled water, dehydrated in a graded ethanol series, and cover-slipped in mounting medium. The expression of various proteins such as p-JNK, COX-2, TNF-α, Caspase 3, Nrf2 and HO-1 were quantitatively determined by ImageJ software 1.8_172 (NIH, USA).

2.9. Acetic acid-induced vascular permeability

To determine the extent of systemic inflammation, vascular permeability test was performed following administration of acetic acid with slight modification [20]. Animals were separated into acetic-acid-induced (Negative control group), (Acetic acid + Mesalazine 10 mg/kg and Acetic acid + Saikosaponin A, 5, 10 mg/kg) treated groups. 1%

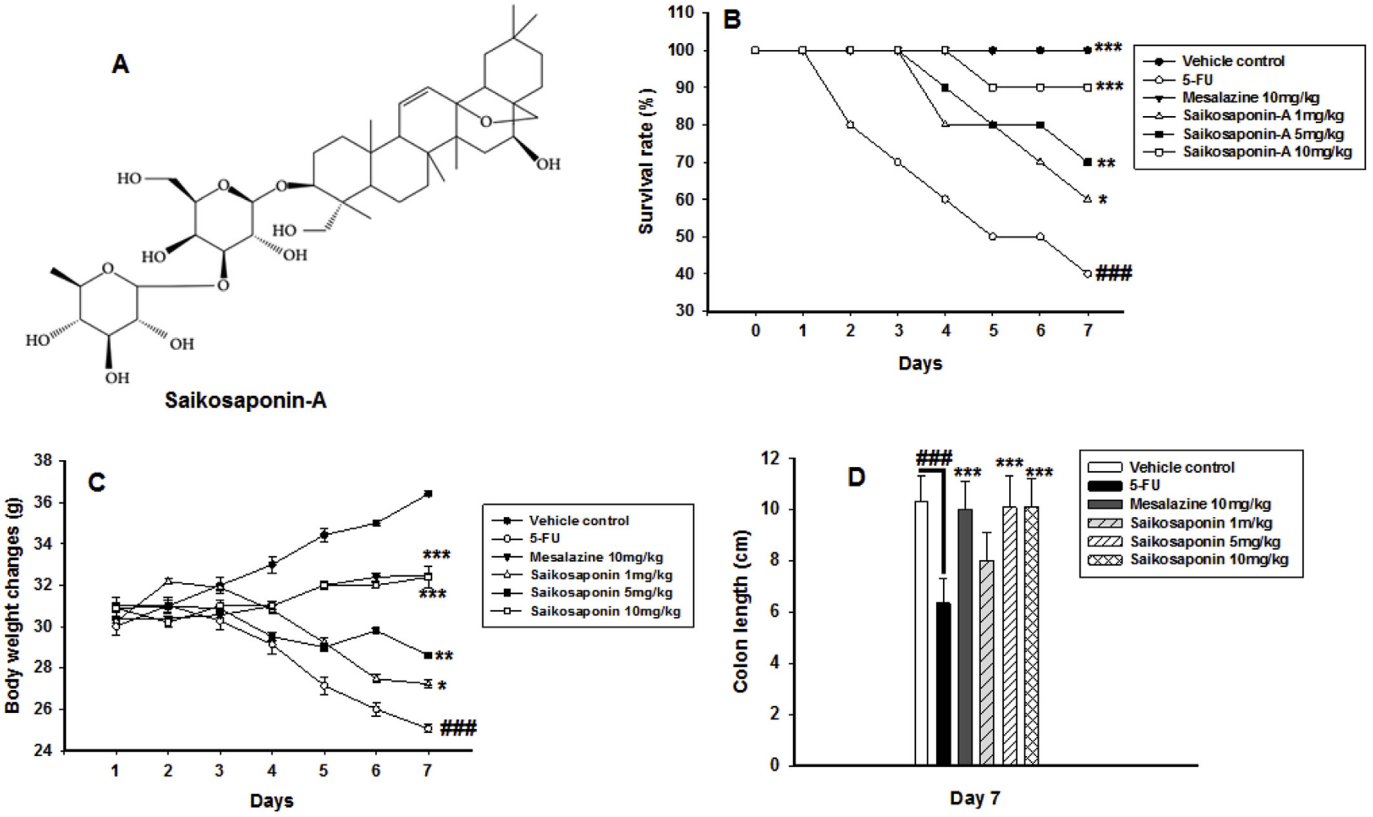

Fig. 3. Effects of Saikosaponin-A pre-treatment on physical manifestations. The structure of the saikospaonin-A (A), Percent survival rate (B), Body weight changes (C) and colon length changes (D). On daily basis, all mice were monitored in case of body weight changes and survival. Data were expressed as the mean ± SD (n = 8 mice/groups). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ indicates significant difference of each treatment as compared to negative control group (only 5-FU). $^{\#\#\#}P < 0.001$ indicates significant difference of negative control group (only 5-FU) as compared to vehicle control group (only normal saline).

Evans blue (10 ml/kg) dissolved in 0.9% normal saline was injected intravenously into the tail vein of all group's animals after 30 min of pretreatment. An (i.p) injection of 0.6% (10 mg/kg) of acetic acid administered to all the groups after 1 h. After 50 min mice were killed by cervical dislocation and 8 ml of normal saline was injected into the peritoneal cavity and again collected these washing solutions in test tubes. In order to clarify turbidity caused by proteins, 80 µl of 1 N NaOH were added to each tube and kept at 4 °C over the night. Along with these samples Evans blue concentration in intestinal tissue was also observed and analyzed by UV/VIS spectrophotometer (UV-2800 Lumsail, China) at 610 nm.

2.10. NO determination

For the determination of NO concentration in plasma, animals were sacrificed at the end of experiment and blood was collected in EDTA tubes, centrifuged at 500 × *g* for 10 min. Serum obtained was assayed for NO determination using Griess reaction [21]. Briefly, 50 µl of blood plasma and 50 µl of normal saline with an equal volume of Griess reagent (1% sulfanilamide in 0.1% naphtylethylenediamine dihydrochloride and 5% phosphoric acid in distilled water) were mixed and absorbance was determined by using microplate reader at 540 nm, with absorbance co-efficient calibrated using standard sodium nitrite solution.

2.11. Estimation of anti-oxidant enzymes (GSH, GST, catalase and SOD) concentration in intestinal tissues and oxidative stress marker MDA

Reduction of glutathione was estimated by previously developed method by Moron et al. with slight modification [22]. Activity started by mixing 0.1 ml of tissue supernatant in 2.4 ml phosphate buffer stock solution and 0.5 ml freshly prepared 5, 5-Dithiobis (2-nitrobenzoic acid) DTNB stock solution. Intensity of yellow color developed was measured on spectrophotometer at 412 nm after 10 min. The resultant GSH concentration values were expressed as µmoles of GSH/g of sample. GST activity was measured with its conventional substrate 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm by previously described method of Habig [23]. Catalase activity in intestinal tissue was performed and results were expressed as micromoles of H_2O_2 degraded/mg protein/min, at 240 nm absorbance [24]. The effect of the Saikosaponin-A treatment was investigated on the SOD activity. The SOD assay was performed as reported previously. Briefly, the tris-EDTA (50 mM, pH 8.5), pyragallol (24 mM) and sample were mixed in the 96 well plates (200 µl reaction mixture) and the absorbance was noted at 420 nm. All the activities were performed in triplicates. Furthermore, lipid peroxidation (LPO) rate was estimated according to protocols reported by Utley et al. via estimating the Malondialdehyde (MDA) concentration with some modification [25]. The assay mixture consists of 580 µl of 0.1 M phosphate buffer (PH 7.4), 200 µl supernatant, 20 µl of mM ferric chloride, 200 µl of 100 mM ascorbic acid and incubated on water bath at 37 °C for 60 min. After 1 h of incubation reaction was stopped by adding 1000 µl of 10% trichloroacetic acid (TCA) and 1000 µl of 0.66% thiobarbituric acid (TBA) to samples. Tubes retained on water bath for 20 min, then cooled on ice bath and finally centrifuged at 3000 × *g* for 10 min. Supernatant absorbance and blank (containing all reagents except test sample) was measured at 535 nm in order to determine the concentration of thiobarbituric acid reactive substances (TBARS), which was expressed as nM TBARS/min/mg protein.

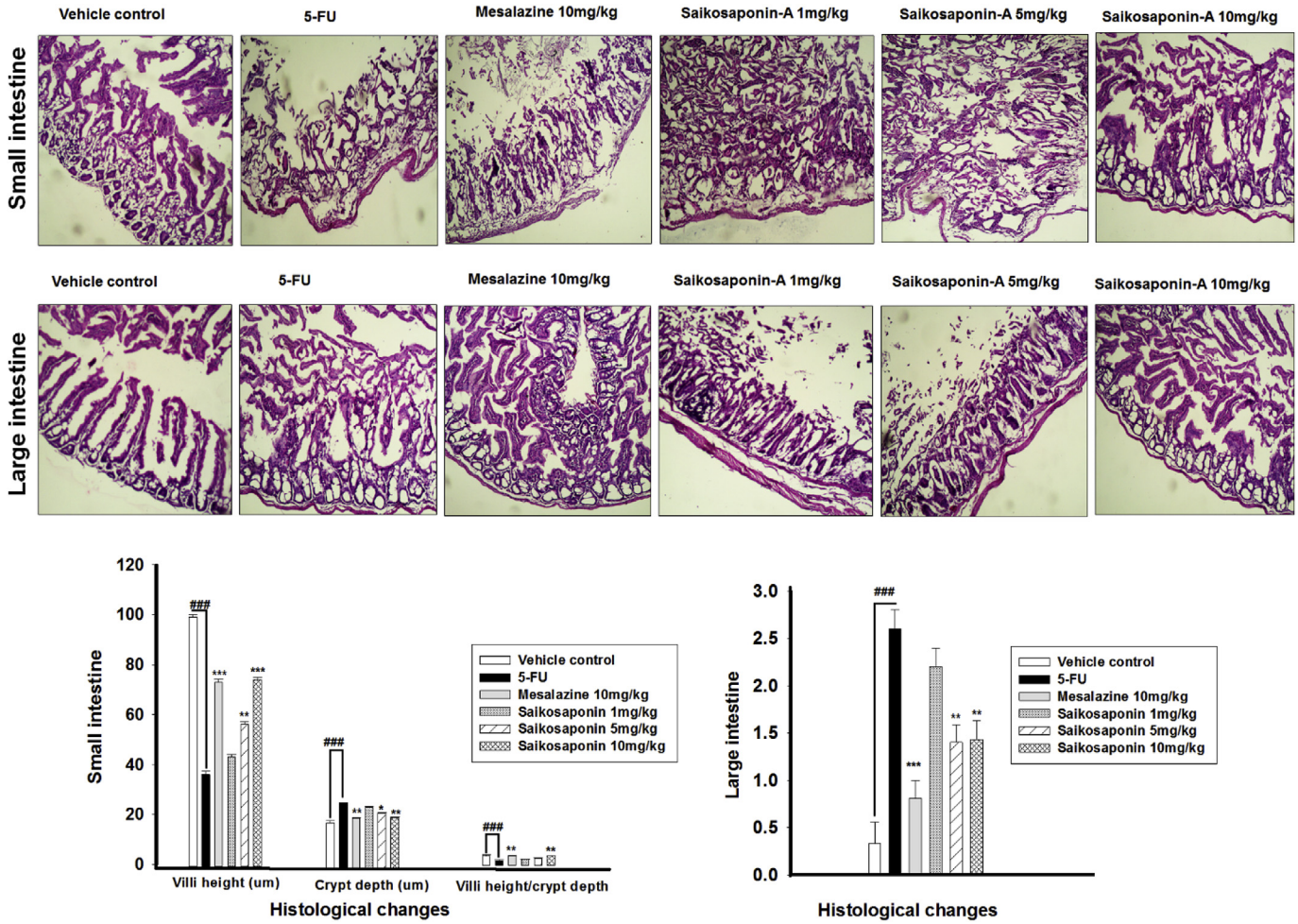

Fig. 4. Effect of Saikosaponin-A on (H & E) staining for both small and large intestinal histological features in mice administered with 5-FU. Photomicrographs were taken at (10X) magnification of both small (4) and large intestinal jejunums (4) across all groups. Vehicle control group represent no histopathological changes with tall and intact villi. While, 5-FU treated group represent remarkable histopathological changes followed by severe epithelial atrophy of villi, enormous inflammatory cells infiltration within lamina propria, vacuolization and edema both in mucosa and muscularis. Treatment with Mesalazine and Saikosaponin-A (1, 5 and 10 mg/kg) significantly reduce the histopathological abnormalities. Data were expressed as the mean ± SD (n = 8 mice/groups). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ indicates significant difference of each treatment as compared to negative control group (only 5-FU). $^{\#\#\#}P < 0.001$ indicates significant difference of negative control group (only 5-FU) as compared to vehicle control group (only normal saline).

2.12. Measurement of inflammatory cytokines

Pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) in intestinal tissue were determined by commercially available ELISA assay kits (eBioscience, Inc., San Diego, CA) according to protocols narrated by Khalid [26].

2.13. Measurement of complete blood picture

At the end of experiment, blood was taken by direct cardiac puncture and kept at 4 °C in EDTA tubes. Different blood cells were counted by using SYSMEX XE-2100 Hematology Analyzer (Sysmex, Kobe Japan).

2.14. Serum electrolytes analysis

Blood samples were collected in EDTA tubes and were centrifuged at 3000 × *g* for 5 min. The serum was collected in fresh sterile tubes and examined for sodium, potassium and bicarbonate by Department of Pathology (Excel-lab, Islamabad, Pakistan) [27].

2.15. Alteration of luminal microflora environment and culturing of fecal samples

To determine changes of luminal microflora in BALB/c mice after treatment of 5-FU, two type of selective media were used to isolate specific bacterial colonies in fecal samples of different mice groups. MRS (De Man, Rogosa and sharp agar, Sigma Aldrich) media was used for selective identification of *lactobacilli spp.* While, MS (MacConkey sorbitol, Sigma Aldrich) media for *Escherichia. Coli spp.* Whole methodology and grading system for bacterial colony formation was validated according to previously described methods [28].

2.16. Molecular docking studies

A study used to predict orientation between ligand and protein targets with the help of different docking software. We used Auto Dock Vina software (Auto Dock Vina 1.1.2) for molecular Docking along with PyRx [19]. E value (Kcal/mol) was used for the determination of best docked pose for both ligand and target protein complex by providing binding free energy and binding constant for docked ligands [29]. 3D-structure of ligand (Saikosaponin-A) was prepared in Discovery Studio Visualizer (DSV) and saved as protein data bank (PDB) format. 3D-

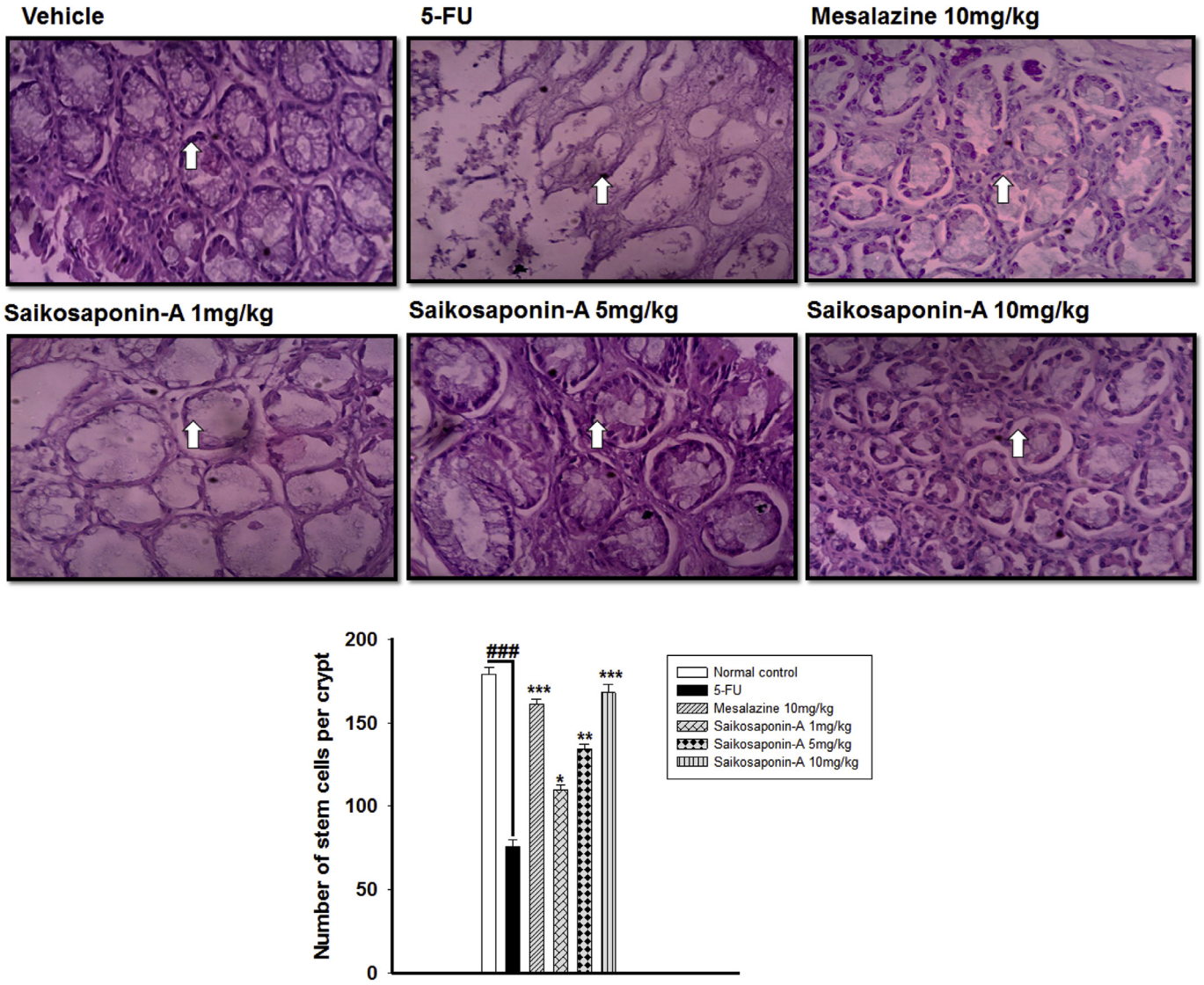

Fig. 5. Effect of Saikosaponin-A on crypt mitotic stem cells of intestinal tissue. Photomicrographs were taken at (10X) magnification with scale bar = 50 μm, representing stained stem cells of intestinal crypts. (A) Vehicle control group representing normal number of stem cells (arrow) in crypt with no apoptotic index. 5-FU group representing complete loss of stem cells (arrow) in crypt with intense apoptotic index. Mesalzine group depicting renewal of stem cells (arrow) in crypt with suppressed apoptotic index. While, Saikosaponin-A administration causes usual proliferation of stem cells (arrow) in crypt with reduced apoptotic index in a dose dependent manner.

structure of targeted proteins (TNF-α (PDB ID:1TNF), COX-2, p-JNK (PDB ID:5AWM), and Casp-3 (PDB ID:3DEI) were download from http//www.rcsb.org/pdb/home/home.do and then purified by Biovia Discovery Studio Client 2016. Post docking interactions for hydrogen bonds, van der Waals and alkyl were determined through (DSV).

2.17. Statistical analysis

Results were expressed as mean (n = 8) ± standard deviation (S.D). Statistical significance between vehicle control and negative control was analyzed with student's *t*-test, while One-way analysis of variance (ANOVA) followed by Dunnett's test was used to test effect of each treatment with respect to negative control group. The *p*-value less than or equal to 0.05 was considered to be statistically significant.

3. Results

3.1. Effect of Saikosaponin-A on physical manifestation (weight loss, food intake, diarrhea induction and severity, survival rate)

All the mice were examined for physical symptoms of mucositis during the whole experimental period. Normal control group mice having smooth fur, normally eating, drinking and locomotor activities followed by no incidence of diarrhea as compared to other five groups. The 5-FU group mice were observed with diarrhea, decrease food intake, reluctance to move, loose hair on day 2 which become more significant ($P < 0.001$) on day 3, 4 and 5 when watery diarrhea with blood followed by arched back was observed. Pre-treatment with Saikosaponin-A shows significant amelioration of physical symptoms in a dose dependent manner (1 mg/kg, 5 mg/kg, and 10 mg/kg) but Saikosaponin-A (10 mg/kg) shows prominent effects as shown (Fig. 2A, B, and C). Intraperitoneally (i.p) treated mice with 5-FU (50 mg/kg) had severe body weight loss from day 3 to day 7 due to excess of diarrhea when compared to normal control group mice, which gained

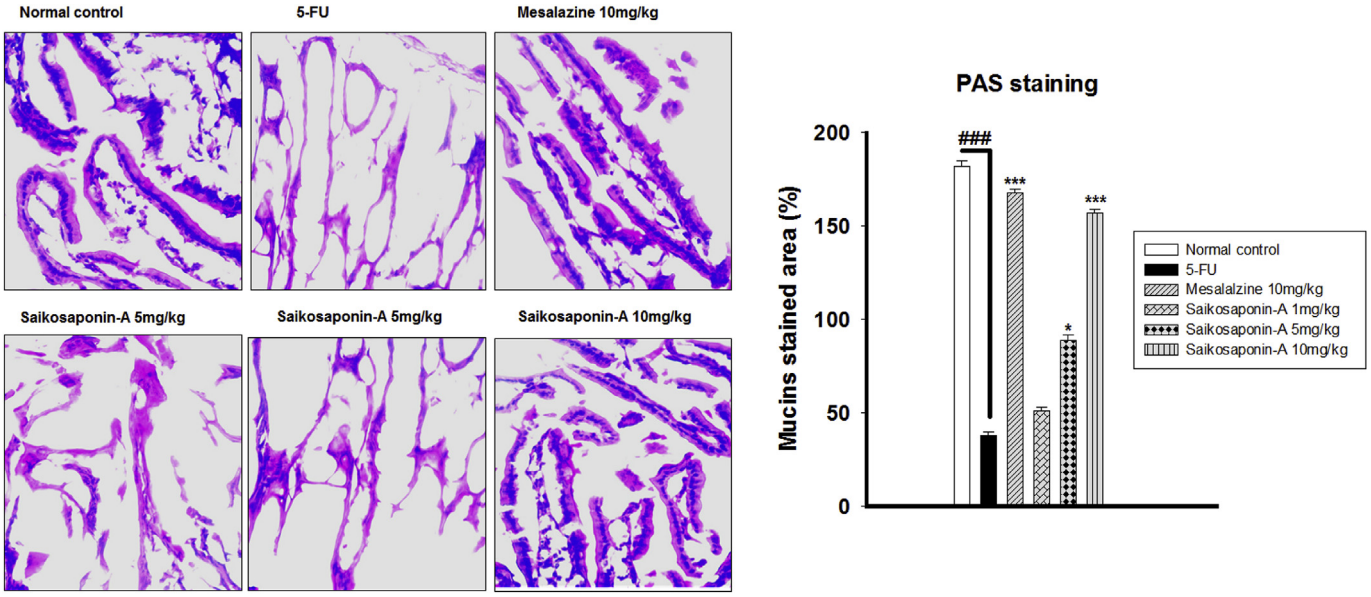

Fig. 6. Effect of Saikosaponin-A on mucin discharge in goblet cells induced by 5-FU in small intestinal tissue. Photomicrograph of Alcian Blue-PAS stain were taken at (10X) magnification with scale bar = 50 μm. (A) Vehicle control group showing normal mucin distribution with intact goblet calls as represented by arrows, (B) 5-FU group reveals complete depletion of mucin (arrow) with increased number of cavitated goblet calls. (C) Mesalazine group depicting reduction of mucin (arrow) depletion with decrease number of cavitated goblet cells. (D, E) Saikosaponin-A (1,5 mg/kg) groups represents moderate depletion of mucin (arrow) with average number to total goblet cells in villus. (F) Saikosaponin-A 10 mg/kg group depicting significant inhibition of mucin layer depletion with suppressed number of cavitaed goblet cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

weight gradually. The physical symptoms associated with the 5-FU administration were markedly improved by the Saikosaponin-A, and the effect was almost similar to the positive control group (Mesalazine 10 mg/kg) (Fig. 3C).

In normal control and positive control groups (Mesalazine 10 mg/kg) no animal death was reported during the entire study. As a whole, 10 deaths were reported in the whole study due to severe diarrhea and dehydration. The percent survival rate was highest in Saikosaponin-A (10 mg/kg) that was 87.5%. The survival rate in case of Saikosaponin-A (5 mg/kg) was 75%, Saikosaponin-A (1 mg/kg) was 62.5% and the lowest survival rate was recorded in 5-FU group mice, which was 50% (Fig. 3B). Notably, treatment with Saikosaponin-A 10 mg/kg significantly ($P < 0.01$) inhibited the shortening of villus length, crypt depth which was further verified by ratio of villus height/crypt depth (Fig. 4C). These findings suggests better protective effect of Saikosaponin-A intestinal mucosa in 5-FU induced Mucositis (Fig. 4).

3.2. Effect of Saikosaponin-A on intestinal histoarchitecture

In vehicle control group there was no histopathological changes (histopathological score: 0). The villi were intact with many tall columnar cells, goblet cells and normal mitotic crypt cells. While, 5-FU treated group mice showed significant histopathological changes in small intestine (histopathological score: 3) followed by complete loss of crypt cells, severe epithelial atrophy of villi, enormous inflammatory cells infiltration within lamina propria, vacuolization and edema both in mucosa and muscularis (Fig. 4). In contrast, continuous pre-treatment with Saikosaponin-A attenuated the morphological changes in small intestine characterized by reduction in villus blunting, crypt cells apoptosis, inflammatory cells infiltration with different histological scores (Saikosaponin-A, 1 mg/kg histopathological score: 2.7, Saikosaponin-A, 5 mg/kg histopathological score: 2, Saikosaponin-A, 10 mg/kg histopathological score:1.2), which could be clearly observed in (Fig. 4). The histopathological score for large intestine also shown in (Fig. 4). Similarly, the 5-FU treatment marked showed reduction of the intestinal crypt mitotic stem cells, however, the pre-treatment with Saikosaponin-A exhibited markedly improvement in the mitotic stem cells dose dependently as evident from the (Fig. 5). The alcian blue PAS-staining revealed drastic decrease in the distribution of the mucins with loss of goblet cells. However, the Saikosaponin-A treatment was associated with significant improvement of the mucins production and showed decrease number of cavitated goblet cells (Fig. 6). For the quantification of the histological changes Image J software 1.8_172 (NIH, USA) was used.

3.3. Effect of Saikosaponin-A on 5-FU-induced depletion of antioxidant enzymes (GSH, GST, catalase and SOD) and lipid peroxidation (MDA level) in intestinal tissue

Anti-neoplastic agents tend to produce oxidative stress through the production of peroxides and free radicals resulting in depletion of antioxidant enzymes, damaging proteins and lipids of cellular components [30]. 5-FU administration caused prominent decrease in concentration of antioxidant enzymes (GSH, GST, Catalase and SOD) in intestinal tissue, which was remarkably enhanced by Saikosaponin-A treatment (Fig. 7). MDA a product of phospholipids peroxidation causes severe stress in cells [31]. 5-FU caused a significant increased level of MDA as compared to normal control group ($p < 0.001$). Attenuative potential of Saikosaponin-A was further reinforced by estimating MDA level which was significantly decreased ($p < 0.001$) providing effective role of Saikosaponin-A in oxidative stress (Fig. 7).

3.4. Effect of Saikosaponin-A on pro-inflammatory cytokines production

Pro-inflammatory cytokines have significant role in the signal amplification stage of mucositis [5]. 5-FU administration resulted in remarkable elevation of cytokines in tissues which was assayed by ELISA Kits (eBioscience Inc, San Diego, CA, U.S.A). Pre-treatment with Saikosaponin-A and Mesalazine both decreased the production of pro-inflammatory cytokines remarkably (Fig. 8A, B, and C).

3.5. Effect of Saikosaponin-A on intestinal microbiota

Mucin have special association with mucositis both by providing

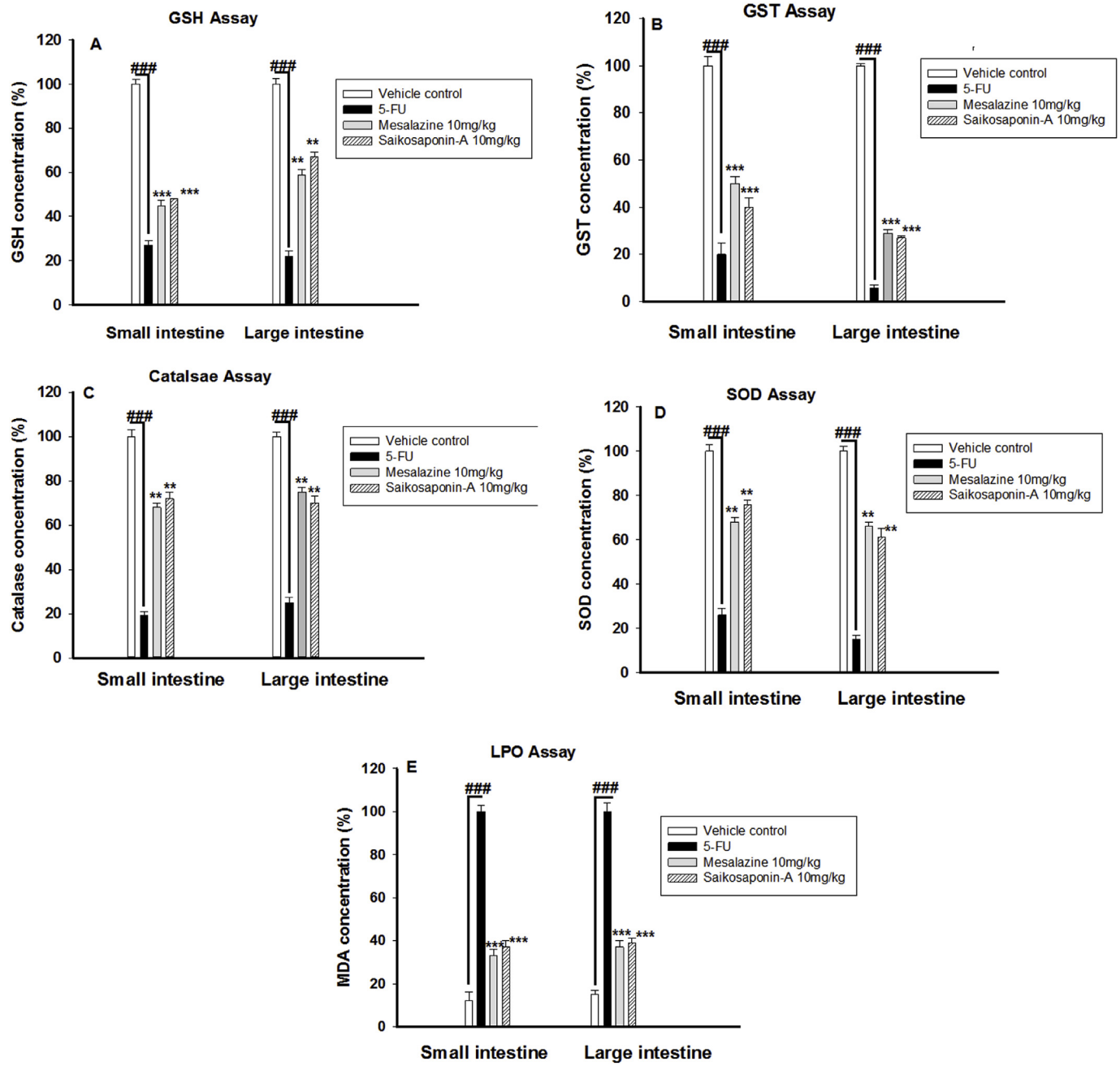

Fig. 7. Effect of Saikosaponin-A on intestinal antioxidant potential. Small and large intestinal GSH level (A). Small and large intestinal GST level. (E, F) Small and large intestinal Catalase level. Small and larg intestine SOD level (D) Small intestinal MDA level (E). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ indicates significant difference of each treatment as compared to negative control group (only 5-FU). $^{\#\#\#}P < 0.001$ indicates significant difference of negative control group (only 5-FU) as compared to vehicle control group (only normal saline).

attachment site for beneficial bacteria and epithelial barrier for pathogenic strain (*E. coli*) to proliferate [28]. 5-FU treatment resulted in complete depletion of mucin in goblet cells, resultantly increasing the number of cavitated goblet cells as compared to vehicle control group ($p < 0.0001$). Conversely, treatment with Saikosaponin-A and Mesalazine significantly restored the level of mucin in goblet cells (Fig. 6). Genes for encoding and composition of mucin is regulated by lactobacillus *spp*. and their products [32]. Administration of 5-FU causing decreased the level of commensal bacteria in negative control group due to excess of diarrhea and increase colonization of harmful strain as result of ulcer in colonic tissue. Pre-treatment of Saikosaponin-A prominently increased the level of beneficial bacteria and decreased the counts of pathogenic strains of *Escherichia coli* (Fig. 9A and B).

3.6. Effect of Saikosaponin-A on 5-FU-induced nitric oxide production

NO, a multidimensional free radical and key mediator of inflammation, causes tissue injury, nerve damage and edema [33]. To evaluate the effect of Saikosaponin-A on NO production in 5-FU-induced mucositis, nitrite concentration was measured using Griess reagent method. 5-FU treatment resulted in significant release of NO production as compared with vehicle control group. In contrast, Saikosaponin-A remarkably suppressed the production of NO in a concentration depended manner ($p < 0.001$) (Fig. 8D).

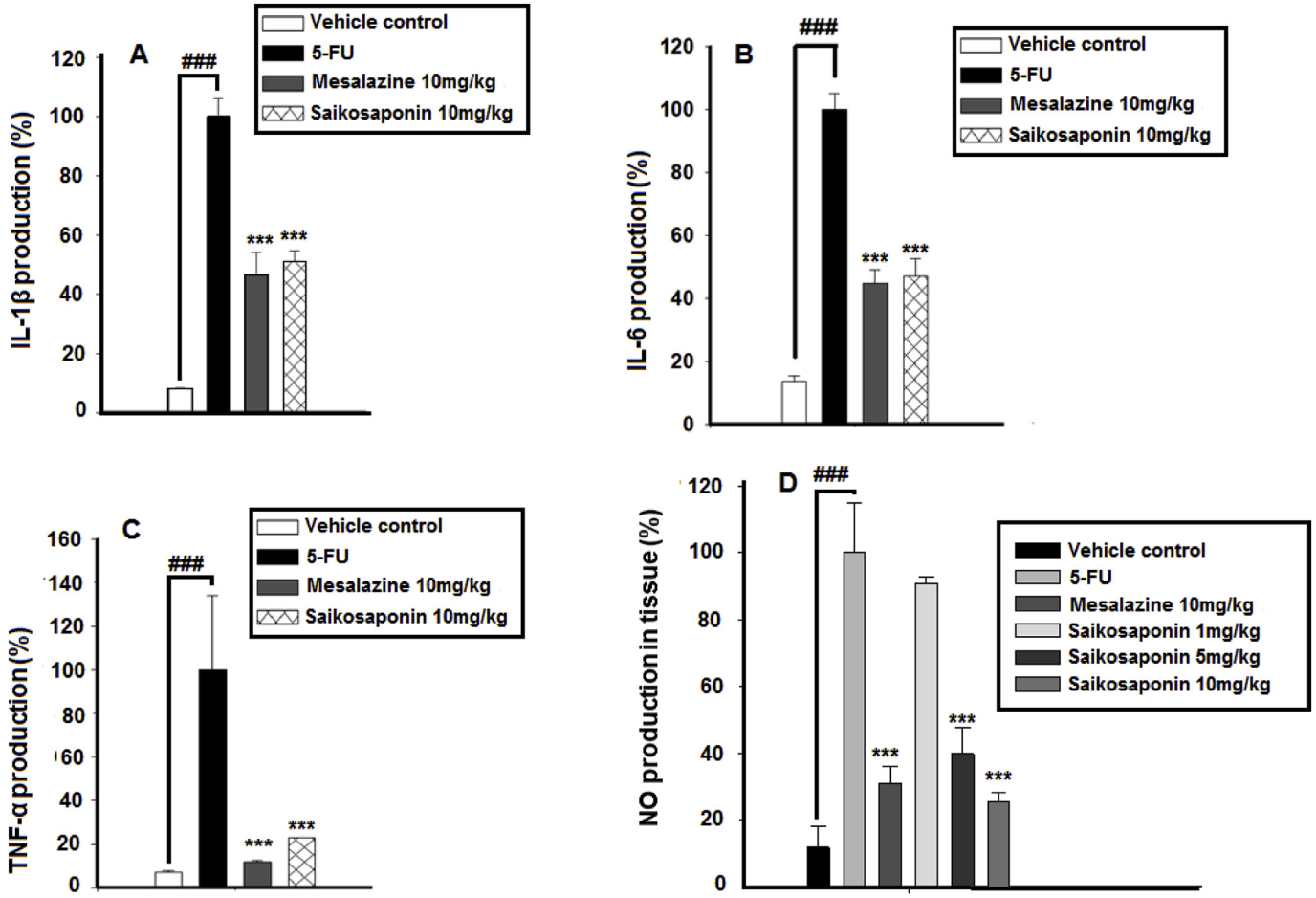

Fig. 8. Effect of Saikosaponin-A on pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) release in intestinal tissue. (A) TNF-α, (B) IL-1β and (C) IL-6 levels in small intestine after 5-FU administration. (D) Effect of Saikosaponin-A on nitrite level in small intestine. Data were expressed as the mean ± SD (n = 8 mice/groups). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ indicates significant difference of each treatment as compared to negative control group (only 5-FU). $^{\#\#\#}P < 0.001$ indicates significant difference of negative control group (only 5-FU) as compared to vehicle control group (only normal saline).

3.7. Effect of Saikosaponin-A on electrolyte profile

Altered electrolytes levels were observed in 5-FU group as compared to normal control group. Serum sodium level was lower in 5-FU group due to less absorptive function of villi as a result of gastrointestinal mucosal damage. While, there was a significant increase in bicarbonate ion as a result of increased amount of CO_2, a metabolite of 5-FU. Saikosaponin-A treatment maintained the electrolyte profile of sodium and bicarbonate level as compared with 5-FU group mice. But, there was no significant difference in potassium level among all the group (Table 1).

3.8. Effects of Saikosaponin-A on expressions of (TNF-α, COX-2, p-JNK, Casp-3, Nrf2 and HO-1) in intestinal tissue using immunohistochemistry

Activated JNK is linked to mucosal apoptosis via caspase activation due to significant overexpression of JNK in intestinal mucosa [34]. In order to strengthen the mucoprotective effects of Saikosaponin-A, we further performed immunohistochemistry for expression analysis of molecular markers of inflammation. 5-FU treated mice showed significant immunoreactivity of TNF-α, COX-2, p-JNK and Casp-3 as compared to normal control group (Fig. 10). Notably, treatment with Saikosaponin-A reversed the activation and remarkably reduced the expression levels of TNF- α, COX-2, p-JNK and Casp-3. The immunohistochemistry was performed to assess the influence of the Saikospaonin-A on the expression of the Nrf2 and HO-1 protein in the 5-FU-induced intestinal tissue. The Saikosaponin-A markedly induced the expression of the Nrf2 and HO-1 proteins following induction of the mucositis with the 5-FU. The mesalazine treated group also enhanced the expression pattern of these anti-oxidant proteins compared to the 5-FU-induced group as evident (Fig. 11). The expression of these proteins were quantified using ImageJ software 1.8_172 (NIH, USA).

3.9. Effect of Saikosaponin-A on Leucocyte count

The mean WBC count for normal saline group was (7.26×10^9/L ± 0.168), 5-FU treated group was (1.23×10^9/L ± 0.185), Mesalazine treated group was (4.69×10^9/L ± 0.195) and Saikosaponin-A 10 mg/kg treated group was observed (3.68×10^9/L ± 0.21). The difference of four groups were statistically significant ($p < 0.05$) (Table 2).

3.10. Effect of Saikosaponin-A on acetic acid-induced vascular permeability

Acetic acid-induced acute inflammation and specifically vascular integrity of intestinal tissues was assayed by Evans blue dye. Maximum vascular permeability was observed in negative control group (only treated with acetic acid). Administration of Saikosaponin-A significantly reduced the vascular integrity evident by Evans blue concentration. Similarly, Mesalazine also remarkably reduced the Evans blue leakage into tissue as compared to acetic-acid induce group (Fig. 12A, B, and C).

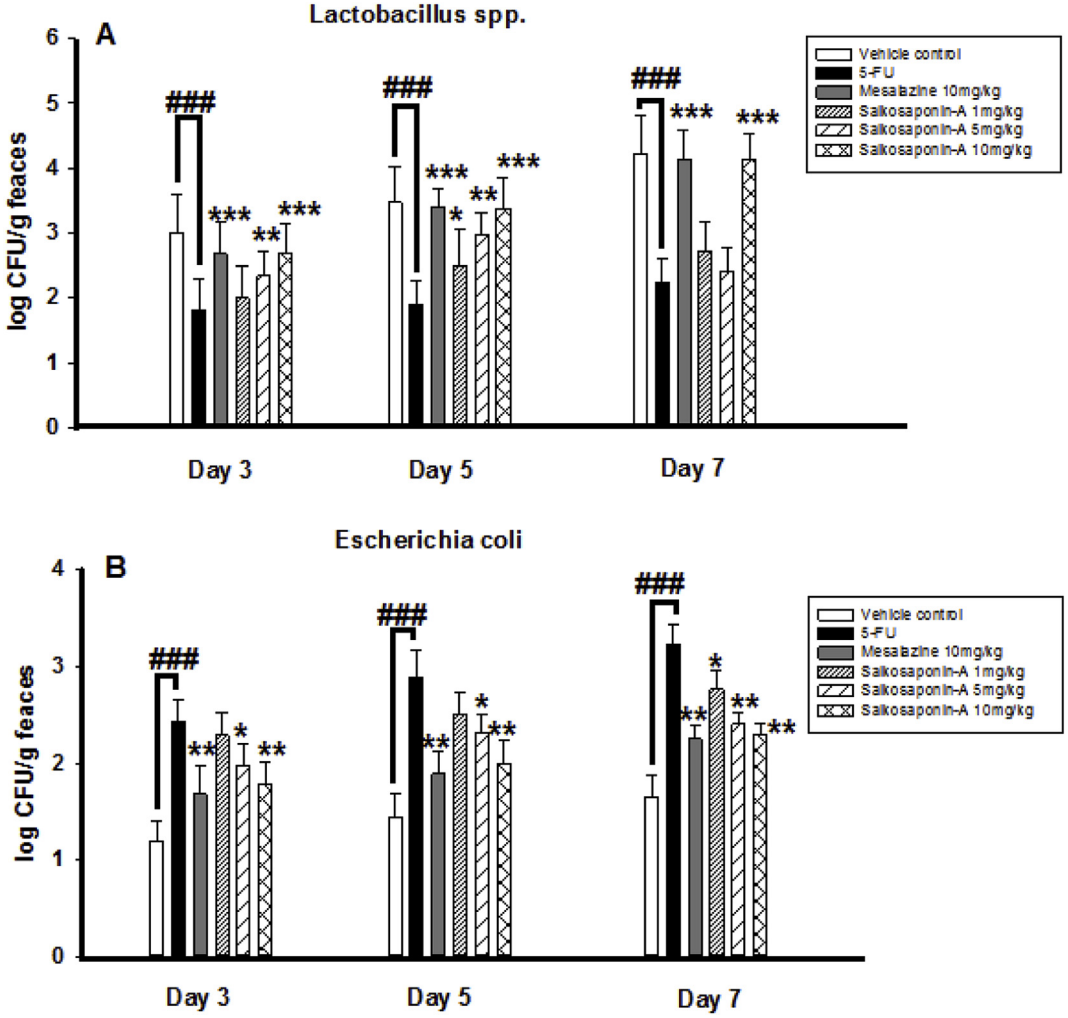

Fig. 9. Effect of Saikosaponin-A on bacterial community of both commensal and pathogenic type. Feces were collected on day (3rd,5th and 7th) and bacterial colonies were plotted as power of log value of colony forming units. (A) lactobacillus spp. presence in mice feces. (B) *Escherichia coli* presence in mice feces.

Table 1
Electrolyte profile.

Sample	Sodium (mEq/L)	Potassium (mEq/L)	Bicarbonate (mEq/L)
Vehicle Control	153. ± 0.458	8.933 ± 0.404	31.5 ± 0.5
5-Flourouracil (50 mg/kg)	143.533 ± 0.611$^{###}$	10.033 ± 0.152$^{##}$	34.666 ± 0.577$^{###}$
Mesalazine (10 mg/kg)	149.933 ± 0.0577*	9.17 ± 0.923	32.166 ± 0.35
Saikosaponin-A (10 mg/kg)	148.566 ± 0.513*	9.95 ± 0.14	32.333 ± 0.56

Data is shown as mean ± SEM (n = 8). $^{###}P < 0.001$ vs Normal saline *P < 0.05, **P < 0.01 and ***P < 0.001 vs 5-FU.

3.11. Molecular docking approach of Saikosaponin-A for COX-2, Casp-3, p-JNK and TNF-α

Saikosaponin-A was docked against COX-2, Casp-3, p-JNK, and TNF-α, while the results were expressed as binding energy values. The cumulative interactions involved in binding of Saikosaponin-A were conventional hydrogen bond, carbon hydrogen bond, alkyl bond and van der Waals. Additionally, Saikosaponin-A formed three hydrogen bonds (two hydrogen bonds with LYS 66 and one hydrogen bond with ARG 344) of p-JNK. Beside this, Saikosaponin-A also formed electrostatic and van der Waals interactions to further confirm binding stability with p-JNK (Fig. 12A and B). Similarly, Saikosaponin-A formed four hydrogen bonds (two hydrogen bonds with ASP 453 and two hydrogen bonds GLN 449) of COX-2. Other members like, PRO 162, LYS 169 and ASP 173 had electrostatic and van der Waals interactions with Saikosaponin-A (Fig. 13C and D). Saikosaponin-A also best fit with Caspase-3 (Fig. 13E and F) and TNF-α (Fig. 13G and H) with all types of polar and electrostatic interactions as visualized by DSV. While the binding energy of the Saikosaponin-A with their targets are listed in the (Table 3).

4. Discussion

Anti-neoplastic therapy used for various type of malignancies trigger structural and functional changes in gastrointestinal tract [35]. 5-FU used as anti-neoplastic agent, especially in the treatment of breast and GIT cancer [36]. Its perpetual use resulted in adverse effect, particularly intestinal mucositis causes preliminary cessation of treatment which impact patient quality of life [37]. The noticeable effects of 5-FU in GIT includes apoptosis of rapidly dividing cells of basal epithelium,

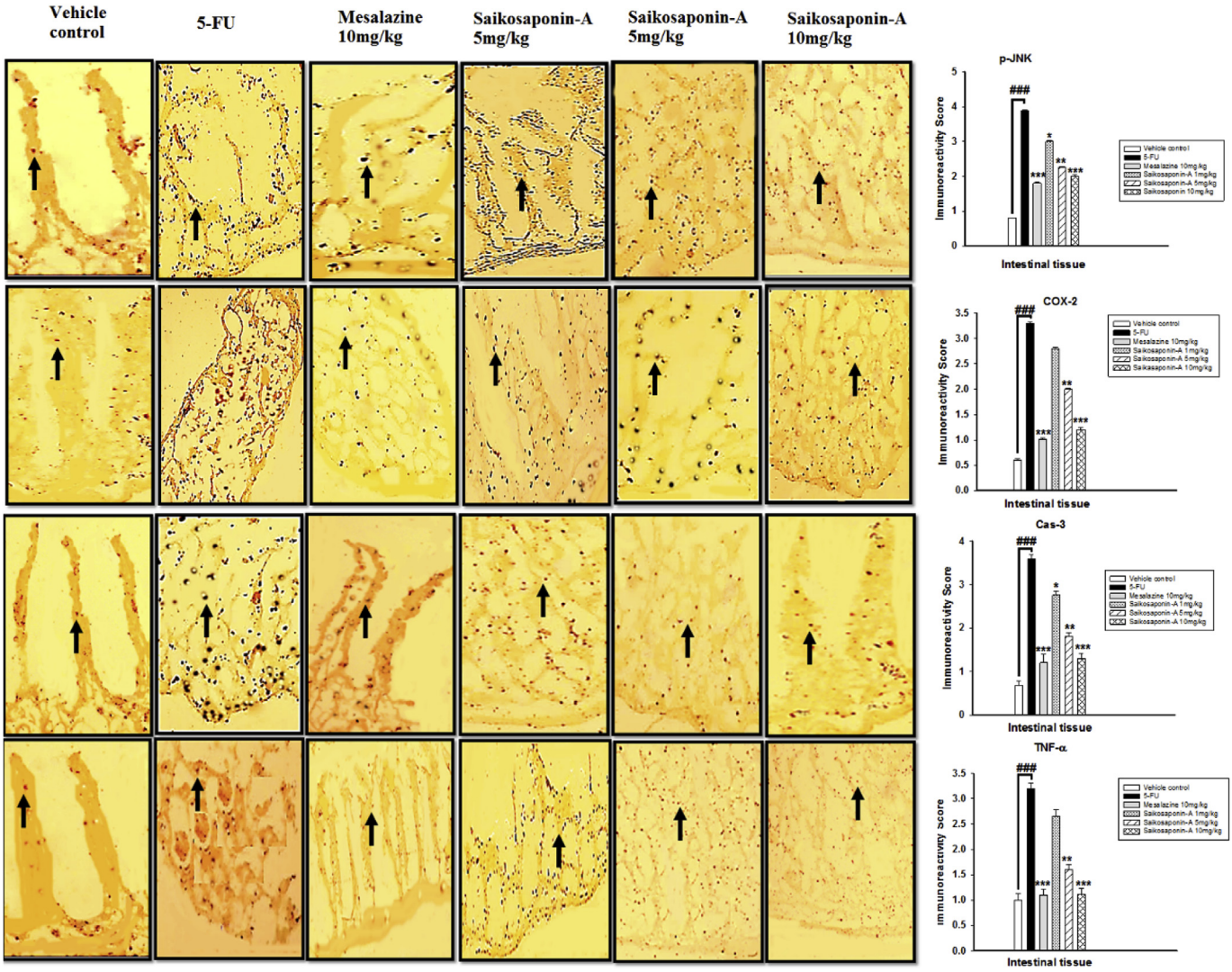

Fig. 10. Effect of Saikosaponin-A on 5-FU-induced activation of pro-inflammatory and pro-apoptotic markers in mice intestinal tissue. Photomicrograph (magnification 10X; scale bar = 50 μm) representing immunoreactivity of COX-2, Casp-3, p-JNK and TNF-α in intestinal tissue. 5-FU group represents intense expression of COX-2, Casp-3, p-JNK and TNF- α (brown dots) as compared to Mesalazine and Saikosaponin-A group which show weak immunostaining (brown dots). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

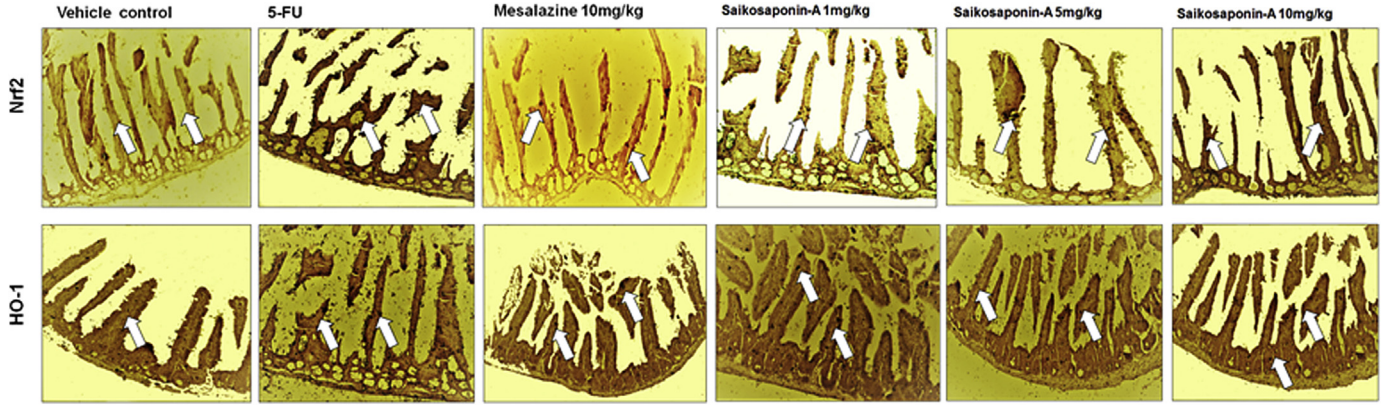

Fig. 11. Effect of Saikosaponin-A on 5-FU-induced activation of the anti-oxidants enzymes such as Nrf2 and HO-1. The Saikosaponin-A dose dependently enhanced the expression of the Nrf2 and HO-1 enzymes following induction of the mucositis with the 5-FU. The mesalazine treated group also should enhanced expression of the Nrf2 and HO-1 compared to the 5-FU-induced group. The immunoreactivity score was quantified for Nrf2 and HO-1.

Table 2
The effect of Saikosaponin-A on blood profile.

Blood Count	Vehicle Control	5-FU (50 mg/kg)	Mesalazine (10 mg/kg)	Saikosaponin-A (10 mg/kg)
WBC (10^9/L)	7.26 ± 0.168	1.23 ± 0.185$^{###}$	4.69 ± 0.195**	3.68 ± 0.21**
Granulocyte(10^9/L)	0.24 ± 0.036	0.86 ± 0.11$^{###}$	0.37 ± 0.032**	0.42 ± 0.025**
Lymphocyte (10^9/L)	6.48 ± 0.19	1.00 ± 0.09$^{###}$	3.38 ± 0.10**	2.92 ± 0.03**
RBC (10^{12}/L)	8.45 ± 0.1	6.42 ± 0.10$^{###}$	8.28 ± 0.02*	7.24 ± 0.04*
Hemoglobin(g/dl)	13.7 ± 0.05	10.1 ± 0.11$^{###}$	13.13 ± 0.11*	12.66 ± 0.05*
Hematocrit (%)	32.46 ± 0.07	56.46 ± 0.55$^{###}$	38.54 ± 0.34**	39.01 ± 0.70*

Data is shown as mean ± SEM (n = 8). $^{###}$P < 0.001 vs Normal saline *P < 0.05, **P < 0.01 and ***P < 0.001 vs 5-FU.

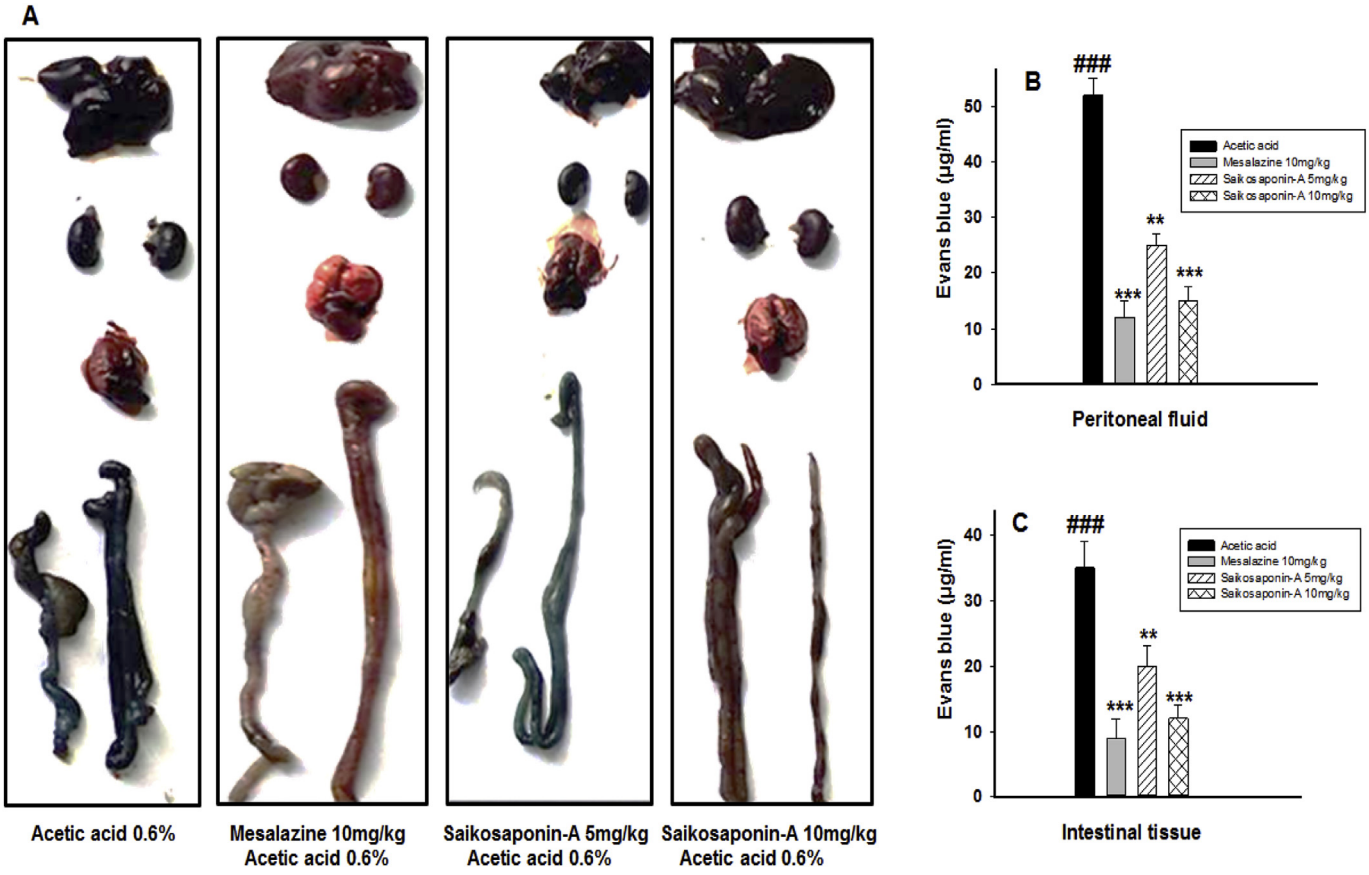

Fig. 12. Effect of Saikosaponin-A on vascular integrity induced by acetic acid. (B) Evans blue concentration in peritoneal fluid. (C) Evans blue concentration in intestinal tissue. (A) Representative pictures depicting extravasation of Evans blue in intestinal tissue in all the treated groups. *P < 0.05, **P < 0.01 and ***P < 0.001 indicates significant difference of each treatment as compared to negative control group (only 5-FU). $^{###}P$ < 0.001 indicates significant difference of negative control group (only 5-FU) as compared to vehicle control group (only normal saline). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

atrophy, thinning and ulceration in epithelium of mucosa, vanishing of crypts and goblet cell, last but not the least enhancing the infiltration of inflammatory cytokines in intestinal tissues [38]. [5]. The lining of intestinal mucosa consists of rapid proliferating cells and are very sensitive to various stimuli such as drugs, bacteria and radiation etc [12]. The inflammatory response comprises of three phases such as apoptotic, regenerative and normalization phases [12]. The apoptotic phase starts within two days of exposure to the radiation or chemotherapy, followed by the regenerative phase. While the normalization phase starts four after exposure to inflammatory insult, which is characterized by the normalization of mucosal lining and crypt growth [12].

In the present study, for the first time the mucoprotective effects of Saikosponin-A against the 5-FU-induced mucositis was demonstrated. According to previous studies, administration of 5-FU for three consecutive days result in severe physical manifestation of IM which was observed as severe diarrhea, decreased in food and water intake, body weight loss accompanied with rough fur and reluctance to move [13]. Administration with Saikosaponin-A alleviated all the physical manifestation induced by the 5-FU administration, suggesting a preferable candidate that apparently protect mucosa. Furthermore, treatment with Saikosaponin-A significantly prevented 5-FU induced intestinal damage such as shortening of villus, disappearance of mitotic crypt cells, increase goblet cells emptying, excess of inflammatory cells infiltration and edema which were confirmed by histopathological assessment. All these histopathological aspects reveal the mutoprotective effects of Saikosaponin-A and restrict the development of 5-FU induced IM.

Anti-neoplastic agents are associated with the generation of the significant elevation of the oxidative stress and these oxidative stress can interact with the several essential components of the cell such as cell membrane, lipids and proteins [30]. The marker of the lipid per oxidation such as malonaldehde are reported to be very high during

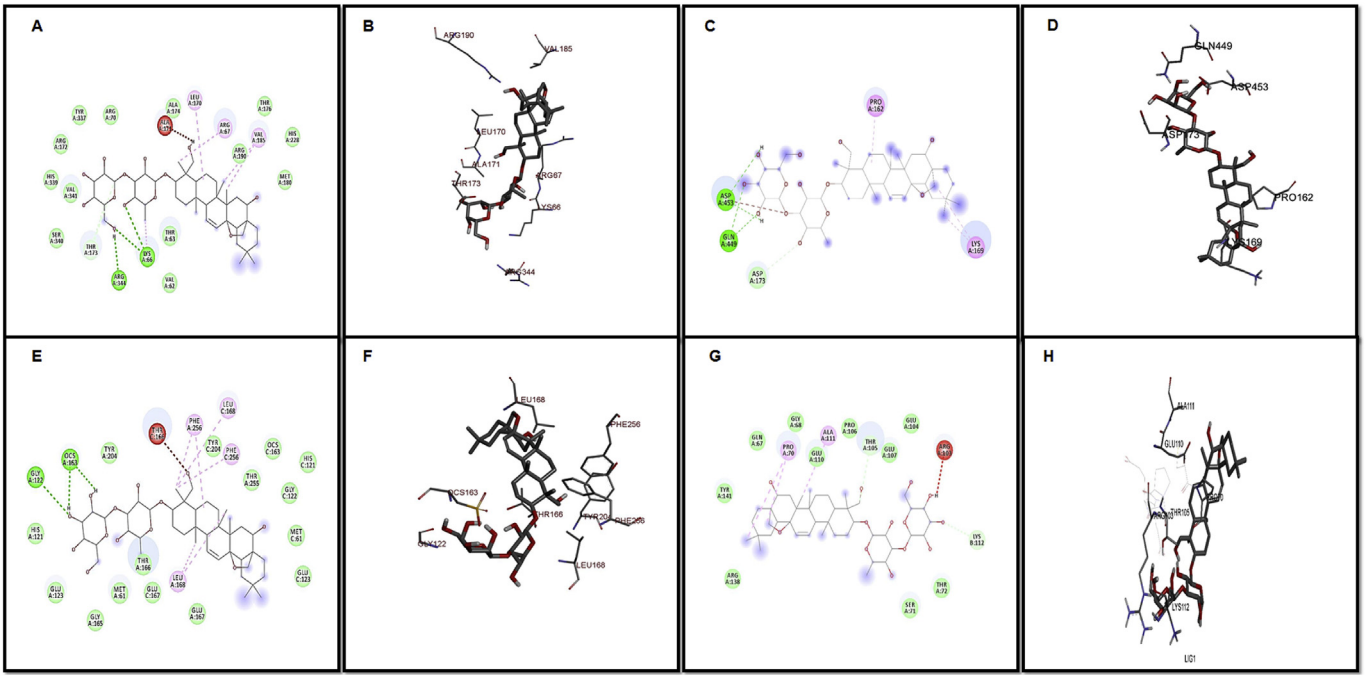

Fig. 13. Effect of Saikosaponin-A on molecular docking with different targets. Saikosaponin-A shows different types of interactions such as Hydrogen bonds, van der Waals and Alkyl-Alkyl interactions with functional groups of different amino acids in both 2D and 3D poses. Interaction between Saikosaponin-A and p-JNK were shown **(A, B)**, COX-2 **(C, D)**, Caspase-3 **(E, F)** and TNF- α **(G, H)**.

Table 3
Binding energies of Saikosaponin-A dock with targets.

Protein Target	Ligand	Binding energy
	(Saikosaponin-A)	(Kcal/mol)
TNF- α	Saikosaponin-A	−6.5
COX-2	Saikosaponin-A	−7.6
Casp-3	Saikosaponin- A	−8.00
p-JNK	Saikosaponin-A	−8.2

inflammatory conditions, and its concentration is related with the extent of oxidative burden [39]. The 5-FU administration can trigger the oxidative stress by suppressing the endogenous anti-oxidants mechanism such as GST, GSH, Catalase and SOD, while elevated level of MDA has also been reported [39]. In the proposed study, the 5-FU administration markedly compromised the anti-oxidants mechanism such as GST, GSH, Catalase and SOD, while the MDA level was raised sharply. The Saikosaponin-A group showed marked increased in the anti-oxidants enzymes, while the level of the MDA was attenuated promisingly. It was found that treatment with Saikosaponin-A not only increase the anti-oxidant enzymes level by repressing ROS production but also reinforce its anti-oxidant potential through inhibition of lipid-peroxidation, suggesting that it increase therapeutic efficacy of anti-cancers. ROS production initiated a series of downstream signaling via pivotal transcriptional factor NF-κB, which regulates several cellular activities like inflammation, cell division, apoptosis, differentiation and development [15]. 5-FU can trigger the activation of the transcriptional factor NF-κB in intestinal tissue and consequently up-regulate expression of several genes especially, those concerned with the production of pro-inflammatory cytokines [40]. The excessive production of the pro-inflammatory cytokines via positive feedback mechanism further activates the NF-κB signaling and facilitate the swift discharge of the mucins from the goblet cells rendering them cavitated [41]. As a result the level of mucin in goblet cells decreases, effecting mucus layer of intestine, subsequently reduce the attachment of the commensal bacteria. While assist the entry of pathogenic bacteria such as *E. coli*, hence effecting available nutrients and all these events leads to increases in ROS production and whole mechanism trigger the 5-FU-induced mucositis [42]. The 5-FU administration increased the expression level of the NF-κB markedly. However, the Saikosaponin-A treated group significantly attenuated the expression of the NF-κB compared to the control. Furthermore, present study demonstrated that pre-treatment of Saikosaponin-A preserved the intestinal microbiota via protecting the intestinal mucosa from the deleterious effect of 5-FU and reduced the severity of the diarrhea.

The 5-FU administration significantly raised the production of the pro-inflammatory mediators such as IL-1β, IL-6 and TNF-α in the intestinal tissue. However, the Saikosaponin-A treated group marginally reduced the production of the noxious inflammatory mediators in contrast to the 5-FU treated group. Similarly, the mesalazine treated group also attenuated the production of the pro-inflammatory mediator compared to the negative control group. Furthermore, NO is an endogenous pro-inflammatory mediator and its level is increased during inflammation [43]. The 5-FU administration has been reported for increasing the concentration of the NO within the intestinal mucosa and its level is induced by the iNOS gene [44]. The reactive oxygen and nitrogen species (RONS) are implicated within the inflammatory microenvironment, while therapeutic intervention have proved very productive in ameliorating the inflammation [44]. In the present study, 5-FU administration significantly enhanced the NO production in both plasma and the intestinal tissue. However, the Saikosaponin-A administration markedly reduced the NO production in both the plasma and intestinal tissue in contrast to the negative control. Similarly, the mesalazine treated group also encouragingly attenuated the NO production compared with the negative control. The role of JNK and caspase-3 induced apoptosis due to chemotherapy with the 5-FU in the intestinal tissue has been reported [34]. In the present study, 5-FU administration markedly enhanced the expression of the JNK and caspase-3 in the intestinal tissue using immunohistochemistry. However, the expression of these proteins were markedly suppressed in the Saikosaponin-A treated group compared to the negative control. Furthermore, the positive control treated group also markedly attenuated the expression of these proteins. The COX-2 and the TNF-α are well known mediators of

the inflammation and have been implicated in the 5-FU initiated chemotherapies in several animals models [45]. In the current study, the 5-FU administration markedly increased the expression of the COX-2 and TNF-α. However, the Saikosaponin-A treated group showed pronounced decrease in the COX-2 and TNF-α expression compared to the negative control.

The hematological alteration have been reported with various chemotherapeutics including 5-FU. The changes in the WBCs count such as leukopenia, neutropenia, decrease in the RBCs and HB content have been documented with the 5-FU mediated chemotherapy [46]. In the present study, the 5-FU administration markedly decrease the WBCs, RBCs and HB content as evident from the hematology Table 2. However, the Saikosaponin-A administration evidently neutralized the blood changes and the level of blood formed content were brought back to normal compared to the 5-FU-induced group [46].

The 5-FU mediated intestinal mucositis are related with the marked alteration in the serum electrolytes and it might be contributed by the induction of severe diarrhea [28]. The various electrolytes that are affected during the IM includes sodium, potassium, while sharp increase in the bicarbonate ions have been demonstrated due to compensatory mechanism [28]. The 5-FU administration marginally decreased the serum concentration of the sodium and potassium, while striking increase in the bicarbonate ions have been revealed. However, the animals treated with the Saikosaponin-A showed marked improvement in the serum eletrolytes compared to the negative control group. Additionally, the animals treated with the mesalazine also showed marked improvement in the serum chemistry as compared to the 5-FU treated group. Moreover, acetic acid-induced inflammation is considered as acute model of visceral inflammation and the permeability of the Evans blue can be used to assess the extent of inflammation. The Evans blue vascular permeability was significantly increased into the peritoneal cavity in the negative control group challenged with the acetic acid only. However, the Saikosaponin-A treated group markedly inhibited the Evans blue permeability and hence, visceral inflammation as compared to the negative control [47].

5. Conclusion

The current study shows that the Saikosaponin-A improved the overall parameters associated with the 5-FU-induced mucositis. The Saikosaponin-A markedly improved the behavioral factors such as diarrhea score, diarrhea induction, food intake, weight loss and survival rate. The biochemical parameters such as inflammatory marker NO, oxidative stress and inflammatory cytokines such as IL-1β, IL-6, TNF-α and COX-2 were markedly attenuated by the Saikospaonin-A treatment. Similarly, anti-oxidant enzymes were significanlty enahanced by Saikospaonin-A. Furthermore, the Saikosaponin-A treatment significantly improved the histological parameters using H nad E staining and Alcian blue-PAS staining. The Saikosaponin-A also markedly inhibited the p-JNK and Casp-3 enzymes compared to the 5-FU-induced mucositis. Furthermore, the molecular docking study shows significant binding efficiency with the various targets such as TNF-α, COX-2, p-JNK and Casp-3 proteins. The present work indicates for the first time that Saikosaponin-A remarkably inhibit 5-FU-induced intestinal mucositis. Our findings related to mucoprotective effects of Saikosaponin-A will hopefully open a new era to reduce deleterious effects of chemotherapeutics on intestinal mucosa and considering Saikosaponin-A as a favorable therapeutic applicant of potential interest.

Declaration of competing InterestCOI

None.

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