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ANTIDIARRHEAL EFFICACY OF AZITHROMYCIN-LOADED SOLID DISPERSION IN *ESCHERICHIA COLI*-INDUCED DIARRHEAGENIC MICE

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Abstract

Azithromycin possesses low aqueous solubility leading to inadequate absorption and poor bioavailability after oral administration. Solid dispersion of azithromycin represented a formulation with enhanced dissolution and antibacterial activity. The study designed to evaluate the *in-vivo* potential of azithromycin-loaded solid dispersion in *Escherichia coli*-induced diarrheagenic (DEC) mice. Diarrhea was induced in Swiss Albino mice by the oral administration of bacterial culture (100 µl of 10^{10} CFU/ml) in high glucose Dulbecco's modified Eagle's medium (DMEM). The *E. coli*-infected diarrheal mice received oral administration of azithromycin-loaded solid dispersion, equivalent doses of pure azithromycin and/or vehicle (DMEM in high glucose) for 3 days; and their effects on diarrheal score, inflammatory markers, and histology of intestinal tissues were observed. Azithromycin-loaded solid dispersion treatment prevented diarrhea and retardation of growth in infected mice more efficiently than the equivalent doses of pure azithromycin. The *E. coli*-infected mice demonstrated soft feces with irregular intervals; however, the nature and frequency of feces were improved with azithromycin-loaded solid dispersion. The increased level of total white blood cells and % of neutrophil was also significantly decreased with solid dispersion of azithromycin. In contrast, pure azithromycin did not alter the counts compared to disease control mice. Furthermore, the colonic tissues of *E. coli*-infected mice showed loss of epithelial integrity with sub-mucosal edema and also associated with increased serum amylase and C-reactive protein levels. However, solid dispersion of azithromycin-treated mice exhibited restoration of colonic tissue structure with a significant attenuation in serum amylase and C-reactive protein levels compared to pure azithromycin and/or vehicle-treated mice. Solid dispersion of azithromycin was more effective against *E. coli*-infected diarrheagenic mice than the equivalent doses of pure azithromycin; and the effect was dose-dependent.

Key words: Azithromycin, Diarrhoea, *Escherichia coli*, Histopathology, Inflammation, Solid dispersion.

Introduction

Escherichia coli (*E. coli*) induced diarrheal infection is one of the most common causes of morbidity and mortality significantly affecting a large number of populations worldwide. Among the medical conditions, diarrhea is the second leading cause of illness; and approximately 1.6 million deaths occur yearly, particularly in developing and underdeveloped countries (Ahs et al. 2010, Wolde et al. 2022). Approximately 90% of diarrheal deaths occurred in South Asia and sub-Saharan Africa. Infectious diarrhea is a significant cause of morbidity in children (Troeger et al. 2018, Leli et al. 2020, Bapanpally et al. 2021) and more than

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25% of deaths occurred in children under 5 years of age. In India an estimated 0.3 million and in Bangladesh 0.5 million children die of diarrhea every year (Death Study Collaborators 2010). In Pakistan, almost 6.4 million cases of pediatric diarrhea are reported annually (UNICEF 2022) and diarrheal illness is the major cause of childhood mortality (60%) (Kahlown et al. 2006, Saeed et al. 2021). According to the World Health Organization (WHO), Pakistan has the highest ratio of infant mortality in Asia from diarrhea (Daud 2017). It is also evident that about 50% of death is associated with persistent diarrheal infection (Bhutta et al. 2005).

Most of the non-pathogenic *E. coli* strains reside harmlessly in the intestine and very infrequently cause disease of the host; however, several pathogenic strains are responsible for intestinal diseases both in healthy and immuno-compromised individuals (Kaper et al. 2004). Both inflammatory and non-inflammatory varieties of diarrhea-causing *E. coli* strains including enteropathogenic *E. coli* (EPEC), enterohemorrhagic (Shiga toxin-producing) *E. coli* (EHEC/STEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), and enteroinvasive *E. coli* (EIEC) were identified (Robins-Browne et al. 2002, Gomes et al. 2016). Non-inflammatory diarrhea caused by enterotoxigenic *E. coli* (ETEC) and inflammatory diarrhea caused by non-inflammatory diarrhea caused by ETEC are manifested by a rapid onset of watery, non-bloody diarrhea of considerable volume with little or no fever, abdominal pain, malaise, nausea, and vomiting as a result of electrolyte loss. However, diarrhea and its related symptoms disappeared naturally within 24-72 hours. The enteroinvasive (EIEC), enterohemorrhagic (Shiga toxin-producing) *E. coli* (EHEC/STEC), and enteropathogenic (EPEC) strains of *E coli*-induced mild to severe inflammation leading to long-lasting diarrheal illness accompanied by fever. Infection caused by a few serogroups (O157, O26) is characterized by bloody diarrhea i.e., hemorrhagic colitis, whereas infection with the *Shigella*-like serogroups manifested as bacillary dysentery i.e., abdominal pain and scanty stool containing blood and mucus (Evans et al. 1996).

In 2004, WHO and the United Nations Children's Fund (UNICEF) recommended the use of low-osmolarity oral rehydration salts (ORS), zinc supplementation, increased amounts of appropriate fluids, and continuous feeding for the treatment (WHO-UNICEF 2004) and management of acute watery diarrhea (Lobenberg et al. 2000). The zinc supplementation at the recommended dose (10 mg/day of elemental zinc for children aged 2-6 months; and 20 mg/day for older children for 14 days) significantly lowers the duration and severity of diarrheal episodes; thus, reducing the morbidity and mortality (Shah et al. 2012, Sathe et al. 2015). Although levofloxacin and ciprofloxacin (single daily dose 500 mg and 750 mg, respectively) are good alternatives for acute watery diarrhea; and also, febrile diarrhea and dysentery particularly caused by Shigella responded by both antibiotics (500 mg once daily with levofloxacin and twice daily with ciprofloxacin for 3 days), but they became less effective owing to the development of resistance to fluoroquinolones, particularly against Campylobacter species (Zhang et al. 2015, Uchakin et al. 2021). Azithromycin (AZ) is preferred as the first line of therapy for the treatment of acute watery diarrhea (500 mg once daily); and is also effective against febrile diarrhea and dysentery (1000 mg once daily) (Zhang et al. 2015, Uchakin et al. 2021). AZ is widely clinically used for the treatment of respiratory tract infections (RTI) such as bronchitis, pneumonia, pharyngitis, tonsillitis, gonorrhea, skin and soft-tissue infections; and also, for the prophylaxis of *Mycobacterium avium Complex* (MAC) infections. Moreover, it is active against *Hemophilus influenza* (*H. influenza*) and is preferred for the treatment of typhoid fever, trachomatis, cervicitis, and chlamydia infections. Furthermore, AZ is recommended for the prophylactic treatment of high-risk endocarditis patients sensitive to penicillin allergy (Zhang et al. 2007, Sweetman et al. 2009).

AZ is a broad-spectrum semi-synthetic macrolide antibiotic that was derived from erythromycin and contains a 15-membered lactone ring with a methyl-substituted nitrogen atom in the ring structure; thus it differs from other therapeutically available macrolide antibiotics (Zhang et al. 2007, Adeli 2016). AZ acts both as bacteriostatic and bactericidal antibiotic depending upon the causative microorganism and the dose of antibiotic; and exerts its mechanism of action through the inhibition of bacterial protein synthesis by

reversibly binding to the 50S ribosomal subunits of 70S ribosome of the sensitive microorganisms (Zhang et al. 2007, Adeli 2014, pubchem.ncbi.nlm.nih.gov). Despite having a wide-spread therapeutic utility, the efficacy of AZ is now questioned. A major disadvantage of AZ is its low aqueous solubility (0.1 mg/mL) results in poor oral bioavailability i.e., only 37% (Aucamp et al. 2015). According to Biopharmaceutics Classification System (BCS), AZ belongs to class II drugs; hence, the drug dissolution is the rate-limiting step during gastrointestinal absorption (Lobenberg et al. 2000, Sathe et al. 2015). Therefore, enhancement of the aqueous solubility of AZ is very important to improve its oral bioavailability. Several formulation techniques including solid dispersion (SD) (Arora et al. 2010, Sekharan et al. 2014, Li et al. 2015), physical mixture (Wadhwa et al 2016), nanosuspension (Zhang et al. 2007), niosomal gel (Zaid et al. 2022), respirable microparticles (Wang et al. 2018), nanoparticles (Mohammadi et al. 2010, Azhdarzadeh et al. 2012, Khan et al. 2016), liposomes (Vanić et al. 2019) have been successful in improving the solubility of BCS Class II drugs. Among the formulation technique, SD has gained much attention because of its simple formulation process and cost-effectiveness (Dhirendra et al. 2009, Siahi-Shadbad et al. 2014, Iyer et al. 2021). AZ-loaded solid dispersion (ASD) with an improved drug release profile have been developed and demonstrated to enhance *in-vitro* antibacterial activity (Monwar et al. 2022). Therefore, the safety and efficacy of ASD formulations were evaluated in *E. coli*-induced diarrheagenic (DEC) mice.

Materials and Methods

Drugs and chemicals

AZ was a generous gift from Square Pharmaceuticals PLC, Bangladesh. Silica (Carplex-80) was obtained from Evonik Pvt. Ltd. (Hanua, Germany). Sodium carboxy methyl cellulose (Na-CMC) was procured from Qualikems Fine Chem Pvt. Ltd. (India). Acetone was purchased from Merck Co. Ltd., Germany. All other chemicals used were of analytical grade. The ASD formulation was prepared by solvent evaporation method as described earlier (Monwar et al. 2022).

Mice model of diarrheagenic *E. coli* (DEC) infection

DEC strains were collected from the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR´B, Mohakhali, Dhaka). Bacterial cultures were prepared from glycerol stocks maintained at -80°C. Cultures were grown in Dulbecco's modified Eagle's medium (DMEM) containing phenol red and placed in a shaking incubator at 37°C until the cultures turned into orange color indicating optimal growth, (OD_{600} -0.6). Finally, cultures were centrifuged at 3500 rpm for 10 min at 4°C; and the bacterial pellet obtained was re-suspended in high glucose DMEM, to get 10^{10} CFU/ml (Ledwaba et al. 2020). To challenge with DEC, an antibiotic cocktail of gentamicin (35 mg/L), vancomycin (45 mg/L), metronidazole (215 mg/L), and colistin (850 U/ml) was administered in drinking water for 3 days to disrupt resident microbiota which was followed by the drinking of normal water for a single day and thus, to ensure removal of residual antibiotics. Finally, infections were induced in mice by the oral ingestion of 100 µl of DEC inoculum (10^{10} CFU/ml/ mouse) in high glucose DMEM using gastric tubes (Ledwaba et al. 2020).

Animals and experimental protocol

Swiss Albino healthy mice were purchased from the Animal Center, Department of Biochemistry and Molecular Biology, University of Rajshahi, Bangladesh. Mice were housed in cages (24±1°C, 60-70% humidity, 12 hours light/dark cycles) acclimated for 7 days, and given standard rodent chow with water *ad libitum*. Mice were healthy and no diarrhea or mucus secretion was noticed during the acclimation period. Animals were cared according to the standard guidelines, and the protocol was approved by the Institutional Animal, Medical Ethics, Biosafety and Biosecurity Committee (IAMEBBC) at the Institute of Biological Sciences [Memo No: 473(18)/320/IAMEBBC/IBSc], University of Rajshahi, Rajshahi, Bangladesh. After

induction of infection, mice were divided into seven groups and each group comprised of five animals. Age-matched normal mice were used as normal control. Mice were subjected to oral administration of pure AZ, ASD, and/or vehicle for 3 days and sacrificed 24 hours after the treatment ceased (Table 1).

Table 1: Different groups of rats treated with ASD, pure AZ and/or vehicle.

Groups (n = 5)	Treatment
Group 1: Normal Control (NC)	Normal control mice received 100 µl of DMEM in high glucose
Group 2: Disease Control (DC)	Disease control mice received 100 µl DEC inoculum (10^{10} CFU/mouse)
Group 3, 4 and 5: Treatment with AZ (12.5, 50, 100 mg/kg/day)	Disease control mice received 100 µl DEC inoculum (10^{10} CFU/mouse) and treated with AZ 12.5, 50, 100 mg/kg, respectively
Group 6, 7, and 8: Treatment with ASD (12.5, 50, 100 mg/kg/day)	Disease control mice received 100 µl DEC inoculum (10^{10} CFU/mouse) with ASD 12.5, 50, 100 mg/kg, respectively

Clinical course

The body weight (BW) and diarrhea were assessed daily starting from day 0, before infection to day 3 post-infection (Table 2). The % changes in BW and diarrhea score were recorded. Diarrhea score was based on the following-4: 0-well-formed pellets; 1- sticky stools adhering in mucosal wall; 2-pasty stools with or without mucus; 3-watery stools with or without mucus secretion; and 4-stools with occult blood (Ledwaba et al. 2020).

Table 2: Calculation of disease activity index (DAI) score.

Score	Weight loss	Stool consistency	Bleeding
0	Normal	Normal (Well-formed pellets)	Not observed
1	1-5%	Semi-soft	Not observed
2	6-10%	Soft	Occult
3	11-15%	Loose	Occult

Disease activity index (DAI) score used to assess *E. coli*-induced diarrheal infection. The DAI score was calculated as follows: DAI = (body weight loss + stool consistency + rectal bleeding)/3; and expressed as the increasing order of severity (0-3).

Total and differential WBC counts

After anesthesia (pentobarbital 30 mg/kg, ip) mice were sacrificed and blood samples were withdrawn directly from the aorta. Total and differential count of WBC were done by an automatic hematological analyzer (BC-2800 Vet; Mindray, Shenzhen, China) (Sugiuchi et al. 2005).

Estimation of serum amylase and C-reactive protein (CRP) levels

Blood samples collected in test tubes were allowed to stand and centrifuged at 4000 rpm for 15 minutes at 4°C. The serum obtained was stored at 80°C for biochemical analysis. Quantitative determination of serum amylase and CRP levels were performed by enzyme-linked immunosorbent assays using a standard protocol (ELISA, USCN, Wuhan, China) (Wu et al. 2016).

Histopathology of mouse colon

For the macroscopic study, the whole colon of mice from each group was excised and the physical appearance and consistency of stool within the colon were observed. The colon was incised longitudinally and rinsed with ice-chilled normal saline. Finally, opened colon tissues were observed for any sign of inflammation i.e.; red spots and ulceration. Colon tissues (Mid distal) from each group of mice were preserved in 10% neutral buffered formalin. The tissues were then embedded in molten paraffin wax and the embedded sections (5 µm) were cut from the mid organ level by a microtome. The slides were deparaffinized in *p*-xylene and rehydrated in the descending grades of ethanol and followed by rinsed under tap water. The specimens were stained with hematoxylin and counterstained by eosin (HE). Finally, viewed under a light microscope with (400x, Olympus IX71, Japan) connected to a computer and analyzed for any pathological changes.

Statistical analysis

Results were expressed as mean ± standard error of means (SEM). Differences between groups were assessed by one-way analysis of variance (ANOVA) followed by Tukey's test. Values $p<0.05$ were considered as statistically significant. In addition, Spearman's rho test was used for correlation analysis. The data was analyzed by using a statistical software package GraphPad Prism 8.2.1 (San Diego, CA, USA).

Results

Stool appearance and diarrhea score

In NC mice the stool was regular in shape and hard pelleted in nature, whereas *E. coli*-infected DC mice demonstrated moderate to severe diarrhea with characteristics of sticky, watery, pasty mucilage and bloody stool (Fig. 1).

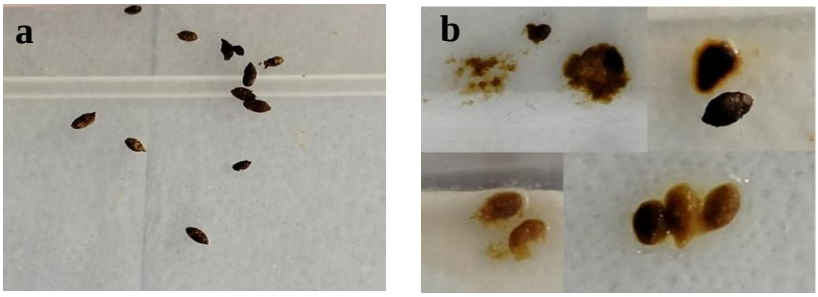


Fig. 1(a-b): Stool appearance of mice. a) normal control, and b) disease control.

Fig. 2 showed a persistent increase in diarrheal score in vehicle-treated DC mice compared to NC mice. Although pure AZ-100 slightly reduced the diarrheal score, the doses AZ-12.5 and AZ-50 did not show any

favorable effects. Mice treated with ASDs prevented diarrhea and the effect was dose-dependent. Interestingly, the effect of ASD12.5 on diarrhea score was comparable to that of pure AZ100 (Fig. 2).

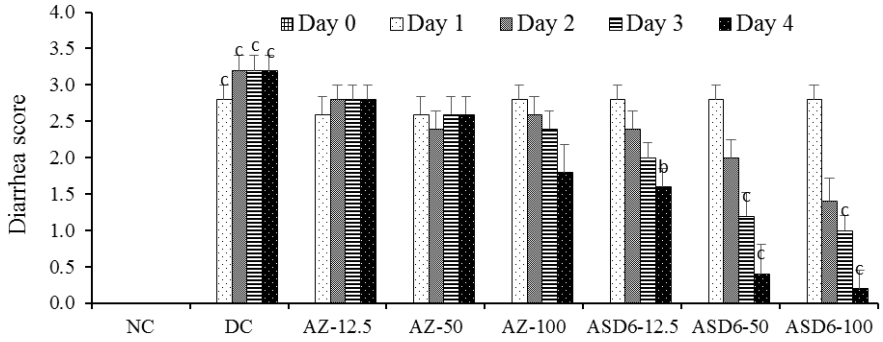


Fig. 2: Effect of ASDs and pure AZ on diarrhea score in DEC-infected mice. **p*<0.05, ***p*<0.01, ****p*<0.001 vs NC, [a]*p*<0.05, [b]*p*<0.01, [c]*p*<0.001 vs DC.

Changes in BW

A significant reduction in BW was observed with vehicle-treated DC mice as compared to NC mice. Oral administration of pure AZ100 partially recovered the BW in infected-mice, a positive increment of BW was observed in ASD-treated mice (Fig. 3).

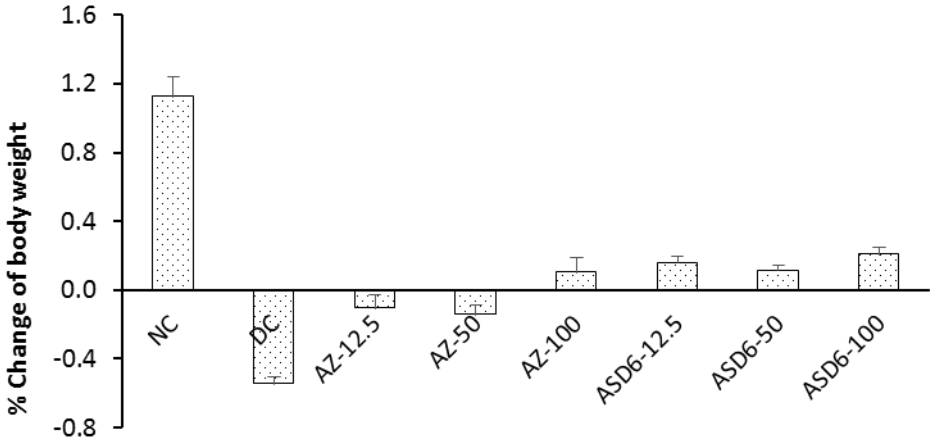


Fig. 3: Effect of ASDs and pure AZ on body weight change in DEC-infected mice.

DC mice exhibited a significantly negative correlation between the % changes in BW and diarrhea score. An overall peak of diarrhea and maximum weight loss was observed on day 4 post-infection. Although, administration of ASDs prevented the weight loss in infected-mice; pure AZ did not (Fig. 4).

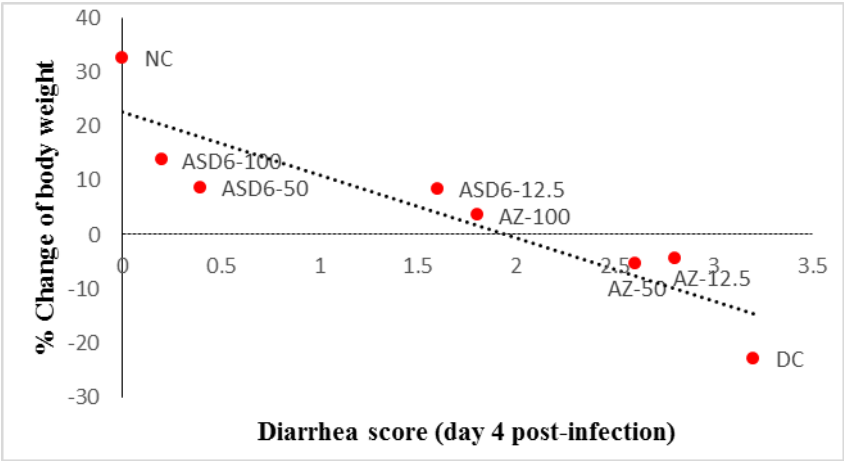


Fig. 4: Correlation between body weight changes (%) and diarrhea score in DEC-infected mice.

Total and differential WBC count

As shown in Fig. 5(a), the total WBC count was higher in DC mice as compared to NC mice. Although, AZ100 treatment attenuated the higher WBC count but no significant changes in WBC count were observed with AZ-12.5 and AZ-50 compared to DC mice. In contrast, ASDs treatment significantly reduced total WBC count; and the effect was comparable to NC mice. A significant increase in the % of neutrophils was observed with DC mice. Although AZ-50, AZ-100, and ASDs treatment group significantly reduced % of neutrophils but group AZ-12.5 did not (Fig. 5b).

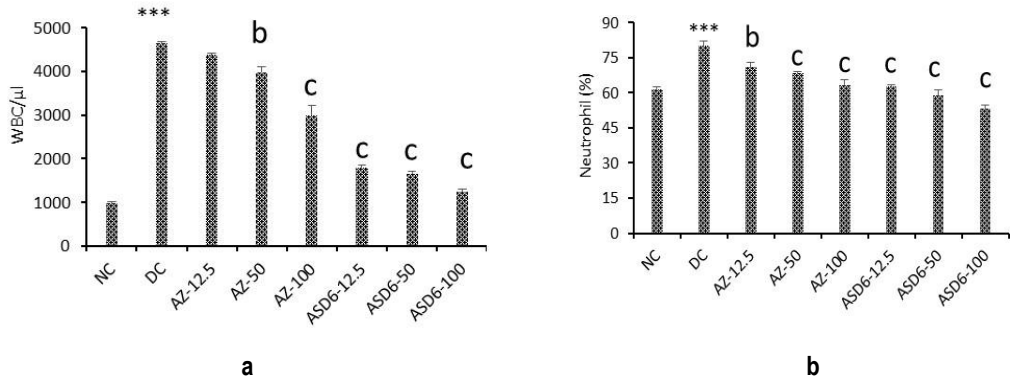
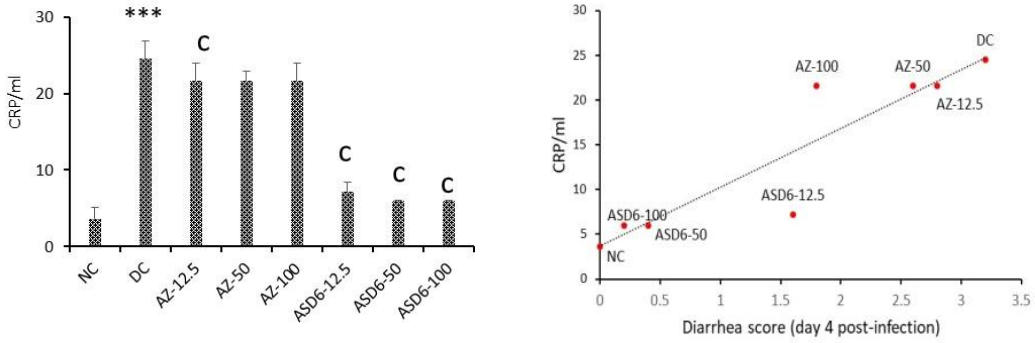


Fig. 5(a-b): Effect of ASD and pure AZ in DEC-infected mice. a) Total WBC count, b) Neutrophil (%). **p*<0.05, ***p*<0.01, ****p*<0.001 vs NC, [a]*p*<0.05, [b]*p*<0.01, [c]*p*<0.001 vs DC.

Serum CRP and amylase

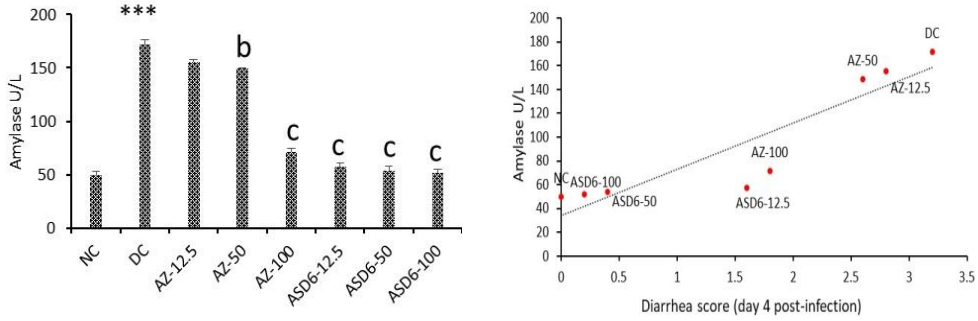
An inflammatory biomarker, CRP level was significantly raised in DC mice and treatment with AZs slightly decreased the CRP value. In contrast, ASDs exerted a significant reduction in CRP levels and the effect was comparable to NC mice (Fig. 6a). A significant positive correlation between CRP level and diarrhea score was observed on day 4 post-infection (Fig. 6b).



a b

Fig. 6(a-b): Effect of ASD and pure AZ in DEC-infected mice. a) Serum CRP levels, b) Correlation between CRP with diarrhea score treated with ASD and pure AZ. **p*<0.05, ***p*<0.01, ****p*<0.001 Vs NC, [a]*p*<0.05, [b]*p*<0.01, [c]*p*<0.001 Vs DC.

Furthermore, **s**erum amylase levels were significantly higher in DC mice (Fig. 7a). Although pure AZ50 and AZ100 showed significant reduction in amylase levels, AZ12.5 did not. Administration of ASDs dose-dependently reduced the serum amylase in infected-mice and a positive association between the serum amylase level and diarrhea score were noted (Fig. 7b).



a b

Fig. 7 (a-b): Effect of ASD and pure AZ in DEC-infected mice. a) Serum amylase levels, b) Correlation between amylase and diarrhea score in DEC-infected mice treated with ASD and pure AZ. **p*<0.05, ***p*<0.01, ****p*<0.001 vs NC, [a]*p*<0.05, [b]*p*<0.01, [c]*p*<0.001 Vs DC.

Histopathology

Fig. 8 shows the macroscopic view of mice colon containing stool inside. In NC mice, the nature of feces was harder and appeared in the regular interval; whereas DC mice represented softer mucilaginous feces with an irregular pattern. However, in ASD-treated mice, the consistency and pattern of stool were improved when compared to the equivalent doses of AZ.

Fig. 8: Macroscopic view of colon in DEC-infected mice treated with ASD and pure AZ.

Fig. 9 shows representative light microscopic images of the colonic tissues from different group of mice. In NC mice villus surrounded by epithelium and crypt; and typical brush border in small intestinal tissues were observed. Fusions in villus structure and decreased crypt structures in intestinal tissues were observed with vehicle-treated DC mice. The impaired appearance of the brush border epithelial cells with damaged crypted area were noticed in mice treated with AZ irrespective of dose. However, numerous typical villi and crypt structures in intestinal tissues were visualized in mice treated with different doses of ASD.

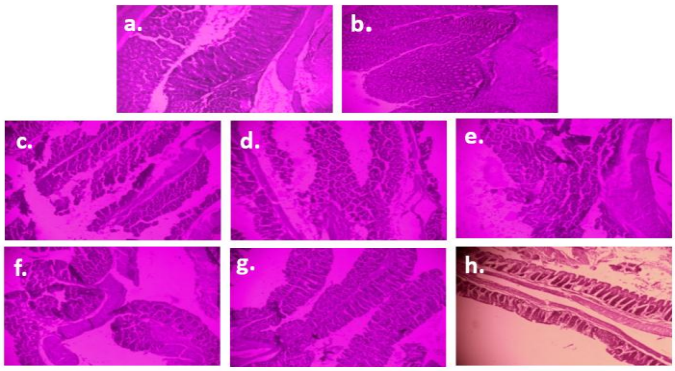


Fig. 9(a-h): Histopathological images of the colonic tissues stained with HE (x400). (a) NC group, crypted villus structure was intact, (b) DC group, DEC-infection caused damage in the colonic tissue characterized by loss of epithelial integrity and submucosal edema, (c-e) Pure AZ treated groups colonic tissue damages persisted irrespective of the dose, (f-h) ASD groups, the colonic tissue regained their structural integrity; and the effect of ASD100 was comparable to normal.

Discussion

AZ is a broad-spectrum macrolide class of antibiotics that generally acts by the inhibition of bacterial growth. Now, it is one of the preferred antibiotics active against sexually transmitted diseases (STD), diarrhea, and upper RTI such as bronchitis and pneumonia, and prevents lungs infections particularly caused by MAC. AZ belongs to a BCS class II drug having high permeability and low water solubility. Thus, AZ is rapidly absorbed and widely distributed throughout the body with an absolute bioavailability of 37%. AZ has a prolonged elimination half-life of 2-4 days which is thought to be due to extensive tissue uptake: and subsequent release of the drug into blood stream. So, due to its higher attainment in tissues, the safety and efficacy of AZ is doubtful. Recently, AZ nanoparticles were prepared by solvent evaporation technique with enhanced solubility and dissolution rate using hydrophilic carriers and also exhibited greater *in-vitro* antibacterial effects (Monwar et al. 2022). Therefore, the safety and effectiveness of newly formulated ASDs were investigated in murine model of diarrhea induced by DEC.

Diarrheal mice represented characteristic soft feces, higher levels of inflammatory markers, and impairment of growth. Also, a significant negative correlation between the BW and diarrhea score was observed. An increase in % neutrophils is associated with acute inflammation after bacterial infection (O'Connell et al. 2015). ASDs treatment significantly improved the nature and pattern of feces; and attenuated total WBC and % neutrophil count whereas AZs did not alter any of the values. Furthermore, a positive increase in body weight and a significant reduction in diarrhea scores were noticed. The results of this study were in accordance with the report where a significant decrease in the number of leucocytes and granulocytes was observed after the treatment with AZ in patients with sickle cell anemia (Uchakin et al. 2021). In the macroscopic view, the DEC-infected mice colon showed soft mucilaginous stool which was normalized after the treatment with ASD. Furthermore, the histopathology of the colonic tissues of DEC-infected mice exhibited loss of epithelial integrity with sub-mucosal edema and increased levels of serum amylase and CRP as described earlier (Matull et al. 2006). In a study, Erben et al. (2014) found that neutrophils interspersing in the epithelial cell layer cause cryptitis due to intestinal inflammation (Erben et al. 2014). Interestingly, Kim et al. (2013) reported a stronger association of CRP levels with inflammatory diarrheal conditions than non-inflammatory diarrheal patients (Kim et al. 2013). After treatment ASDs significantly attenuated the higher serum level of amylase and CRP; and also normalized the colonic tissue structure whereas pure AZ certainly failed to recover the damaged colonic tissue.

Conclusion

ASD was prepared by mixing AZ with carriers i.e., carplex-80 and Na-CMC at optimum ratio (1:3:2) and resulting in increased dissolution rate. The enhanced solubility of AZ was due to conversion of crystalline into amorphous state i.e., providing high surface area that facilitated water adsorption, improved bioavailability and consequently, greater antibacterial activity exhibited by AZ when loaded in SD. Oral administration of ASDs prevented diarrhea, retardation of growth and increased WBC counts better than the equivalent doses of pure AZ in DEC-infected mice. Moreover, ASDs ameliorated colonic tissue inflammation with a significant attenuation in serum amylase and C-reactive protein levels during the progression of diarrhea. So, ASDs were more effective in the treatment of *E coli*-infected diarrheal mice than the equivalent doses of pure AZ. However, further pharmacological investigations are required to elucidate the mechanism of actions of ASDs in DEC-infected mice.

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Conflict of interest: The authors hereby declare no conflict of interest regarding the publication of this article.

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