Synthesis and Ameliorative Effect of Isatin–Mesalamine Conjugates on Acetic Acid-induced Colitis in Rats

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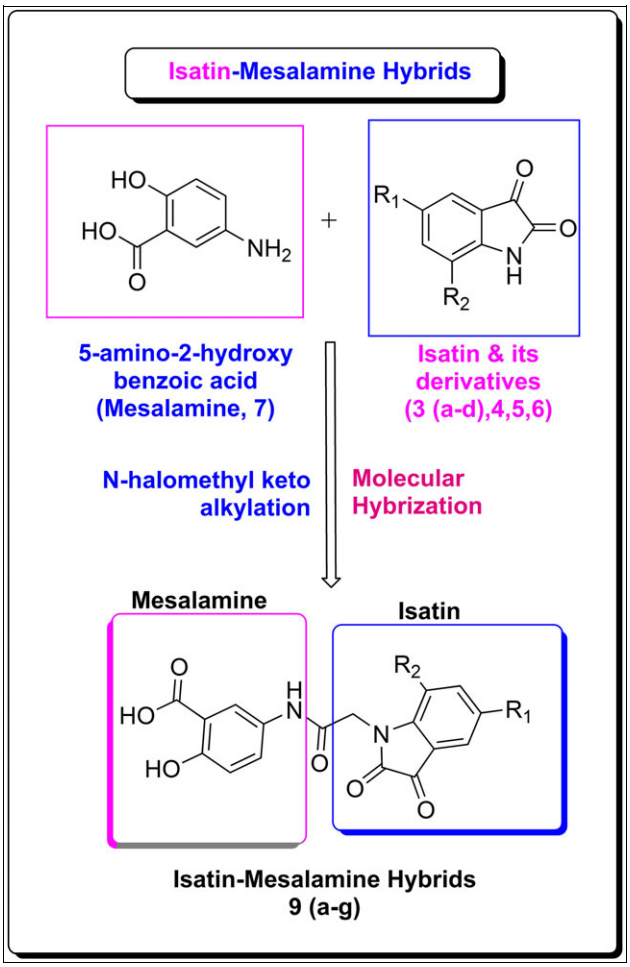


A series of new isatin–mesalamine conjugates (**9a**–**g**) were synthesized *via* conjugation of isatin (**3a**) and its derivatives (**3b**–**3d**, **4**, **5**, and **6**) with mesalamine (**7**) by using chloroacetyl chloride as a bifunctional linker. Compounds **3a**–**3d** were prepared by employing Sandmeyer reaction. Compounds **4**, **5**, and **6** were obtained from isatin (**3a**) *via* previously reported methods. The synthesized compounds were characterized by IR, mass, ^{1}H NMR, and ^{13}C NMR spectral techniques. Synthesized compounds (**3a**–**d**, **4**, **5**, **6**, and **9a**–**g**) were evaluated for *in vitro* antioxidant activity by DPPH assay method using ascorbic acid as standard. Hybrids **9b** (IC_{50} = 368.6 ± 3.5 μM) and **9f** (IC_{50} = 335.1 ± 2.9 μM) showed better antioxidant activity than its parent compounds such as **3a** (IC_{50} = 556.8 ± 2.9 μM), **5** (IC_{50} = 511.9 ± 3.6 μM), and **7** (IC_{50} = 768.9 ± 2.7 μM). Acetic acid-induced ulcerative colitis in rat model was chosen to examine the antioxidant potential of the synthesized hybrids (**9b** and **9f**) in the amelioration of ulcerative colitis. Colonic myeloperoxidase and malondialdehyde enzymes were used as biomarkers of anti-ulcerative colitis activity. In the present study, hybrids **9b** and **9f** reduced the levels of colonic myeloperoxidase and malondialdehyde enzymes significantly ($p < 0.05$) when compared with control (colitic), at a dose (0.03 mM/12.5 mg/kg b.w. p.o.) (50%) less than that of its parent moieties mesalamine (0.16 mM/25 mg/kg) and isatin (0.16 mM/25 mg/kg). Thus, the molecular hybridization was proved to be significant in enhancing the activity of hybrids **9b** and **9f** by reducing the dose.

INTRODUCTION

Ulcerative colitis means inflammation of the colon, caused by the formation of ulcers in the colon and rectum. It is primarily confined to mucosal, sub-mucosal layers of the colon superficially and seldom extends to the distal parts. Ulcerative colitis and Crohn's disease are the two main forms of inflammatory bowel disease whose basic differences include the former (ulcerative colitis) affects only the innermost lining of the colon and rectum, while the latter (Crohn's disease) can occur in all the layers of the bowel walls along the gastrointestinal tract.It is traditionally regarded as the disease of the westernized nations. But the advent of globalization-induced modified dietary habits made this disease to be prevalent across the world. Ulcerative colitis is a chronic recurrent disease, triggered mainly by the excessive immune response that results in the infiltration of polymorphonuclear cells, lymphocytes, monocytes, and plasma cells into the colonic mucosa. Although the exact pathogenesis is unclear, some other etiological factors are responsible for this disease besides the interaction of immune system. Genetic susceptibility, oxidative stress (free radicals generation ROS), drug interactions, and feeding habits are some of the reported etiological factors of the ulcerative colitis [1]. Safe and successful therapies have not been developed so far because of these diverse etiological factors. The current therapy encompasses a variety of therapeutic options depending on the disease progression; such treatments include aminosalicylates, glucocorticosteroids, immunosuppressors, and biological therapies [2,3]. The preferred first line of therapy in the case of ulcerative colitis is the oral/intracolonic administration of mesalamine at relatively high dose of 2.4 g/day. Despite of many drugs available in the treatment of ulcerative colitis, none of them could prevent the relapse of ulcerative colitis. Hence, there is a need to revisit the existing drugs with newer strategies like molecular hybridization.

Molecular hybridization is a unique, economic, and one of the novel drug design and discovery techniques. And it is based on the covalent conjugation of two/more pharmacophoric units or drugs with similar or different biological actions into a single new hybrid with improved affinity and efficacy than their parent moieties [4,5]. Previous literature reports substantiated the role of molecular hybridization in improving the affinity and efficacy of the new hybrids than their parent moieties. The design and synthesis of 4-aminoquinoline–isatin hybrids [6], chloroquinoline–isatin hybrids [7], benzoic acid–isatin hybrids [8], and pomalidomide–isatin hybrids [9] are some of the examples that have demonstrated the efficacy of molecular hybridization. In continuation of these studies and our efforts to synthesize isatin-based hybrids, in this study, we proposed the design and synthesis of isatin–mesalamine hybrids, with an objective to produce newer and effective drugs in the treatment of ulcerative colitis.

Mesalamine, chemically known as 5-amino salicylic acid, belongs to the class of salicylic acid derivatives. It is used in the treatment of ulcerative colitis because it has good antioxidant and anti-inflammatory properties [10]. The generation and release of local reactive oxygen species have long been considered to be involved in the vascular, epithelial, and mucosal inflammatory injury in general and in the pathogenesis of ulcerative colitis in particular. Thus, the potential mechanism of action of mesalamine involves scavenging these free radical moieties and thereby endorsing the role of antioxidants in the treatment of ulcerative colitis [11]. Isatin, an endogenous moiety with wide spectrum of biological activities, was found to have antioxidant properties along with its protective actions against TNBS-induced colitis in rats [12–15]. Numerous literature reports are cited on the role of isatin in the modulation of pro-inflammatory mediators such as cytokine production, reduction of leukocyte infiltration, inhibition, expression, and activity of inducible isoforms of cyclooxygenases that are directly involved in the pathogenesis of the colitis [16]. After reviewing the previous literature reports, it is postulated that covalent conjugation of isatin and mesalamine with similar biological actions would produce new isatin–mesalamine hybrids whose biological activities might be better than either of their parent moieties isatin and mesalamine. The structure–activity relationship incorporated into the design of isatin–mesalamine conjugates (**9a**–**g**) is illustrated in Figure 1. In order to accomplish the assumed hypothesis, isatin–mesalamine conjugates (**9a**–**9g**) were synthesized and primarily evaluated for the *in vitro* antioxidant activity (DPPH method). Later, the conjugates with better antioxidant activity were selected and evaluated further to explore their antioxidant mediated protective effect against acetic acid-induced ulcerative colitis in rat model [17]. Thus, we herein report the protective action of the newly synthesized hybrids against acetic acid-induced ulcerative colitis in rat model.

RESULTS AND DISCUSSION

The present investigation involves the application of molecular hybridization concept in the design, synthesis, and evaluation of isatin–mesalamine hybrids (**9a**–**g**). The synthesis of isatin and its derivatives (**3a**–**d**, **4**, **5**, and **6**) and isatin–mesalamine hybrids (**9a**–**g**) were carried out by adopting previously reported synthetic reactions and methods: such as Sandmeyer reaction, electrophilic aromatic substitution reaction, N-acylation and N-halo

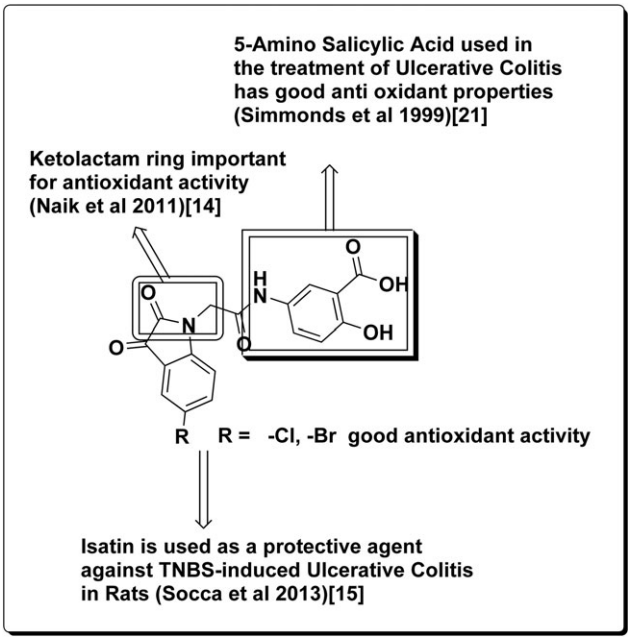


Figure 1. The structure–activity relationship concept incorporated into the design of isatin–mesalamine conjugates (**9a**–**g**).

methyl keto alkylation [18–20]. The synthetic route of all the compounds is summarized in Scheme 1 (**3a**–**d**, **4**, **5**, and **6**) and Scheme 2 (**9a**–**g**). All the synthesized compounds (**3a**–**d**, **4**, **5**, **6**, and **9a**–**g**) were characterized by using the spectral techniques like IR, mass, ^{1}H NMR, and ^{13}C NMR, and their physical data are presented in experimental section.

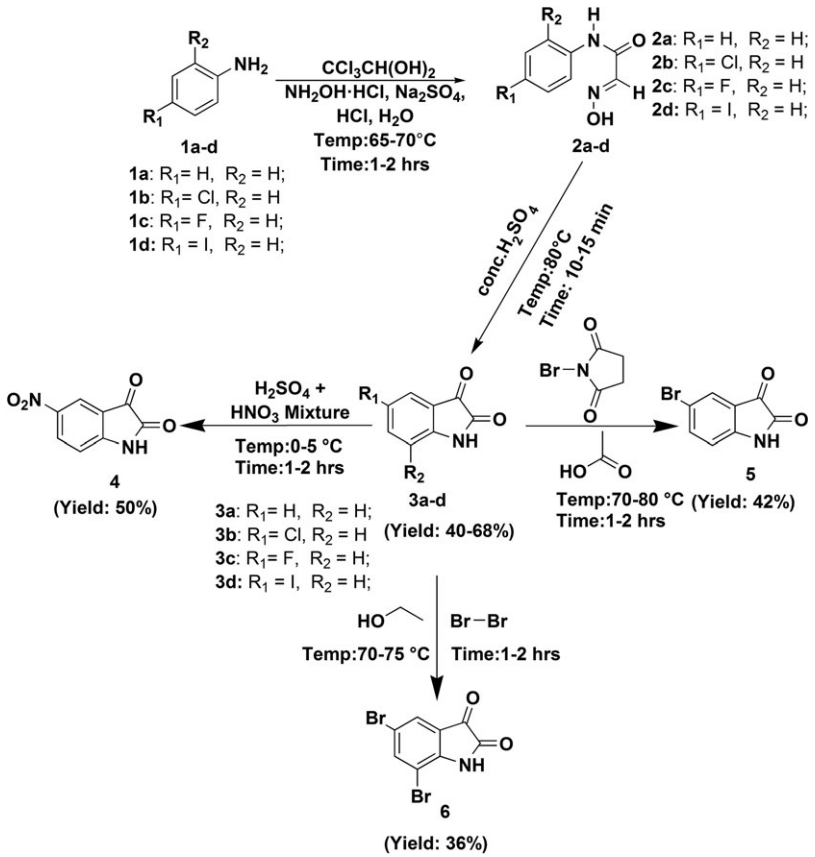
The synthesized compounds (**3a**–**d**, **4**, **5**, **6**) and hybrids (**9a**–**g**) were primarily tested for the *in vitro* antioxidant activity *via* DPPH (1,1-diphenyl-2-picrylhydrazyl radical) assay method as reactive oxygen species is one of the primary causes in the pathogenesis of ulcerative colitis [14,21]. Therefore, the *in vitro* antioxidant activity evaluation study was focused on identifying the potent antioxidants from the set of the synthesized compounds (**3a**–**d**, **4**, **5**, **6**) and hybrids (**9a**–**g**). And the purpose of the aforementioned study was not only to identify the potent antioxidants but also to lead them for further evaluation and correlation of their antioxidant potential in the treatment of ulcerative colitis by using the acetic acid-induced ulcerative colitis in rat model. As known widely, the key parameter to evaluate antioxidant activity is IC_{50}, which is the concentration of the compound required to inhibit 50% of the radical and expressed as micromoles (μM) in this study. All the compounds (**3a**–**d**, **4**, **5**, **6** and **9a**–**g**) displayed moderate to strong antioxidant activity. The IC_{50} values of all the compounds were found to be in the range of 335.1 ± 2.9 μM to 865.1 ± 3.8 μM as shown in Figure 2. The IC_{50} data revealed that the potent antioxidant is synthesized hybrid **9f** (335.1 ± 2.9 μM), and the weak antioxidant is compound **6** (865.1 ± 3.8 μM).

Further, for the sake of structure–activity relationship interpretation, all the synthesized compounds were divided into four categories of compounds: isatin (**3a**), mono-substituted isatins (**3b**–**3d**, **4**, **5**), 5,7-disubstituted isatin (**6**), and isatin–mesalamine hybrids (**9a**–**g**). Molecular hybridization enhanced the antioxidant activity of isatin–mesalamine hybrids (**9a**–**g**) significantly when compared with their parent moieties (**3a**–**d**, **4**, **5**, **6**, and **7**). Substitution of electron withdrawing groups (–Cl, –F, –I, $–NO_2$, and –Br) on isatin-induced variations in the antioxidant activity of the isatin nucleus (**3a**). Flouro (**3c**), iodo (**3d**), and nitro (**4**) substitutions on isatin did not show significant increase in the antioxidant activity whereas chloro (**3b**) and bromo (**5**) substitutions enhanced the antioxidant activity. However, 5,7-dibromo substitutions on isatin decreased the antioxidant activity as opposed to the increase in the antioxidant activity of 5-bromo isatin (**5**). Overall, the aforementioned findings are in accordance with the previous findings reported by Naik *et al*. [14], Socca *et al*. [15], and Simmonds *et al*. [21].

Among all the synthesized hybrids, **9b** and **9f** were found to have better antioxidant activity with IC_{50} values 368.6 ± 3.5 μM (**9b**) and 335.1 ± 2.9 μM (**9f**). As per the design of the experiment, these two active hybrids (**9b** and **9f**) were selected to evaluate their antioxidant potential in the treatment of ulcerative colitis against acetic acid-induced ulcerative colitis in rat model.

Male Wistar rats weighing from 200–250 g were used, and standard animal study protocols were adopted in the present study to examine the antioxidant potential of the

Scheme 1. Synthesis of isatin and isatin derivatives (**3a**–**3d**, **4**, **5**, **6**) from *p*-aniline (**1a**–**1d**) and chloral hydrate.



synthesized hybrids **9b** and **9f** in the treatment of ulcerative colitis and compared them with their parent moieties isatin (**3a**) and mesalamine (**7**). To accomplish this, we used an animal model for ulcerative colitis, developed by Millar *et al*. [17]. According to this model, intra-rectal administration of 2 mL (3% v/v) acetic acid in the rats impaired the colonic macroscopy characterized by changes in colon length, colon weight, and colon thickness in the present study. The changes in various parameters like colonic weight, colonic length, colonic width, colon weight/length ratio, and macroscopic score are presented in Table 1. These parameters are highlighted to illustrate the macroscopic characteristics of ulcerative colitis being produced by the intra-rectal instillation of acetic acid in rats.

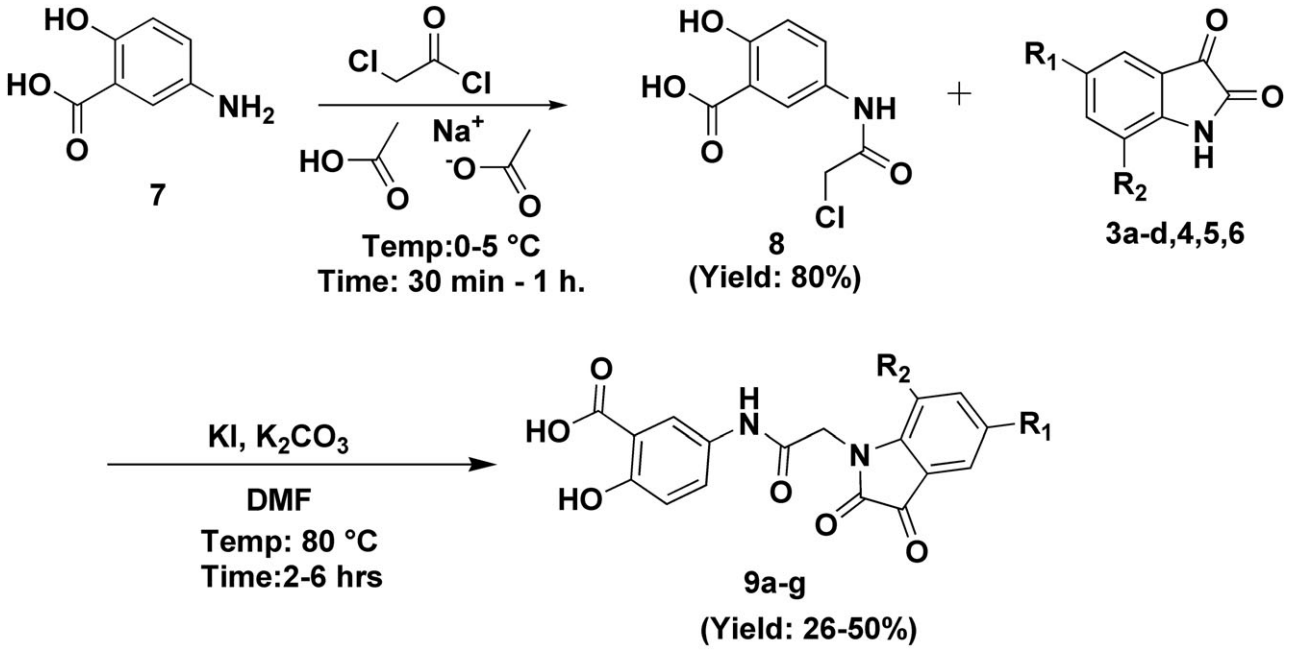
In view of the potent antioxidant activity displayed by the synthesized hybrids **9b** and **9f**, their dose of administration is fixed at nearly half of the dose of their parent moieties isatin (**3a**) and mesalamine (**7**), and the dose is expressed in millimoles (mM) that enables the comparison between synthesized hybrids (**9b** and **9f**) and their parent moieties (**3a** and **7**). Therefore, it is hypothesized that the synthesized hybrids **9b** and **9f** would be effective even at half the dose of their parent moieties isatin (**3a**) and mesalamine (**7**), which gives the scope for reducing the dose of mesalamine whereby minimizing the toxic and side effects as well.

Administration of IS (**3a**, 0.16 mM/25 mg/kg), CLIS-AS (**9b**, 0.03 mM/12.5 mg/kg), BRIS-AS (**9f**, 0.029 mM/12.5 mg/kg), and AS (**7**, 0.16 mM/25 mg/kg) to all the group of rats for 7 days significantly attenuated the alteration in morphological parameters. The data proved that the administration of synthesized hybrids **9b** and **9f** even at half the dose of their parent moieties found to show similar improvement in the morphological parameters same as that of their parent moieties isatin (**3a**) and mesalamine (**7**) whose observations are depicted in Figures 3, 4, and 5.

Ardizzone *et al*. and Budarf *et al*. reported that intra-rectal instillation of acetic acid in rats caused oxidative damage and alteration in oxidative parameters like myeloperoxidase (MPO) and malondialdehyde (MDA) [22,23]. Likewise, in the present study also, intra-rectal instillation of acetic acid in rats elevated the levels of the colonic MPO and MDA enzymes significantly as a sign of oxidative damage in all the groups except naïve.

Although the exact pathogenesis of ulcerative colitis is unclear, oxidative stress is considered as one of the best targets to develop the successful therapy for ulcerative colitis. Oxidative stress is manifested by the reactive

Scheme 2. The synthetic pathway of isatin–mesalamine hybrids (**9a**–**g**).



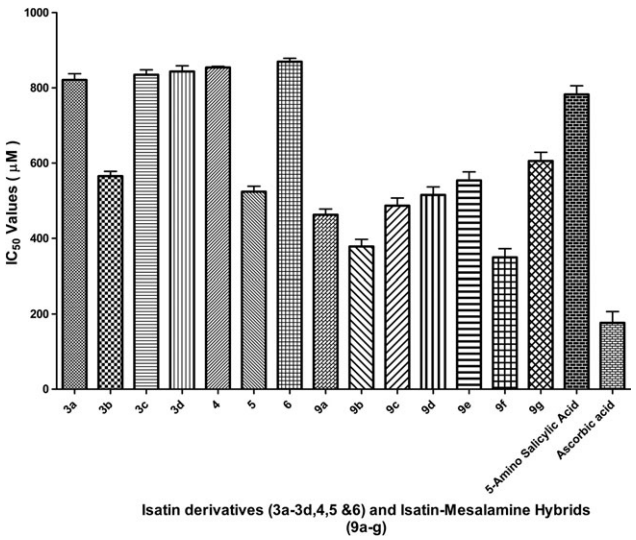


Figure 2. DPPH antioxidant activity data (IC_{50} value in μM) of the isatin derivatives (**3a**–**3d**, **4**, **5**, **6**) and isatin–mesalamine conjugates (**9a**–**9g**).

oxygen species; it impairs cellular membrane stability and eventually causes cell death by lipid peroxidation whose end product is MDA. An increase in free radicals causes overproduction of MPO and MDA [24]. MPO is an enzyme responsible for elevation of oxidative stress. It is present in the gastric tissue and secreted excessively in the inflammatory conditions of the gastric mucosa [25]. Thus, it can be argued that there exists an inversely proportional relationship between the concentrations of MPO and MDA and the antioxidant potential of the

Table 1
Effect of isatin (**3a**), isatin–mesalamine conjugates (**9b**, **9f**), and mesalamine (AS) on morphological alterations in acetic acid-induced ulcerative colitis in rats.

	Naïve (non-colitic)	Control (colitic)	Isatin (0.16 mM/25 mg/kg) (**3a**) p.o. for 7 days	5-Chloroisatin–mesalamine conjugate (**9b**) (0.03 mM/ 12.5 mg/kg) p.o. for 7 days	5-Bromoisatin–mesalamine conjugate (**9f**) (0.029 mM/ 12.5 mg/kg) p.o. for 7 days	Mesalamine (AS) (**7**) (0.16 mM/25 mg/kg) p.o. for 7 days
Colon length (cm)	16.91 ± 0.92	13.07 ± 0.10	16.88 ± 0.28	16.06 ± 0.31	16.51 ± 0.27	17.00 ± 0.10
Colon weight (g)	1.09 ± 0.04	2.17 ± 0.26	1.12 ± 0.09	1.18 ± 0.02	1.14 ± 0.03	1.02 ± 0.05
Colon weight/length ratio (g/cm)	0.06 ± 0.00	0.16 ± 0.05	0.06 ± 0.02	0.07 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
Macroscopic score	0.00 ± 0.00	4.00 ± 1.03	2.10 ± 0.08	2.40 ± 0.06	2.30 ± 0.06	2.10 ± 0.03

Data were analyzed by two-way analysis of variance (non-parametric) followed by Bonferroni test ($n = 6$). The mean ± SD of all groups were found to be statistically significant at $p < 0.01$ when compared with control (colitic) group.

administered compounds (**9b**, **9f**, **3a** and **7**). It means lower the concentration of MPO and MDA, higher will be the antioxidant potential and anti-ulcerative colitis activity of the compounds administered vice versa.

Administration of IS (**3a**, 0.16 mM/25 mg/kg), CLIS-AS (**9b**, 0.03 mM/12.5 mg/kg), BRIS-AS (**9f**, 0.029 mM/ 12.5 mg/kg), and AS (**7**, 0.16 mM/25 mg/kg) to the rat groups for 7 days significantly reduced the levels of oxidative parameters like colonic MPO and MDA enzymes when compared with the naïve ($^{*}p < 0.01$) and control ($^{**}p < 0.05$) groups as shown in Figures 6 and 7. The decrease in the concentration of colonic MPO and MDA enzymes is attributed to the antioxidant potential and anti-inflammatory properties of both the isatin (**3a**) and the mesalamine (**7**) [10,15,16,21].

These observations are testimonial to confirm the role of synthesized hybrids **9b** and **9f** in free radical scavenging property and thereby its use in the amelioration of ulcerative colitis. Thus, the results in the present study provide evidence to an idea that molecular hybridization enhances the potential of the resulting hybrids *via* their action on multiple targets.

EXPERIMENTAL

Animals. Male Wistar rats (230–280 g) were procured from Mahaveer Enterprises, Hyderabad, Telangana, India. They were allowed to have normal laboratory diet with free access to water and maintained in a controlled room (temperature 24–25°, humidity 70–75%, 12 h light:12 h dark cycle) at Animal House Facility, University College of Pharmaceutical Sciences, Kakatiya University, Warangal, Telangana, India. Access to food was restricted for 24 h before the inductions of colitis in rats. There was no restriction upon water access; rats were allowed to have free access to water throughout the study. The experimental protocols designed for the present study was approved by Institutional Animal Ethics Committee (IAEC), U.C.P.Sc, Kakatiya University, Warangal, Telangana, India (IAEC/ 23/U.C.P.Sc/K.U/2015), and the protocols used were in accordance with given specifications and guidelines of the Committee for Control and Supervision of Experimentation on Animals, Government of India.

METHODS

Chemistry. ***General procedure for the preparation of compounds (3a–3d, 4, 5, and 6).*** Preparation of isatins (**3a–3d**, **4**, **5** and **6**) was carried out as per the already reported procedures (mentioned in [18–21,26]).

Preparation of 5-[(chloroacetyl)amino]-2-hydroxybenzoic acid (8). 5-Amino Salicylic acid (mesalamine) (**7**)

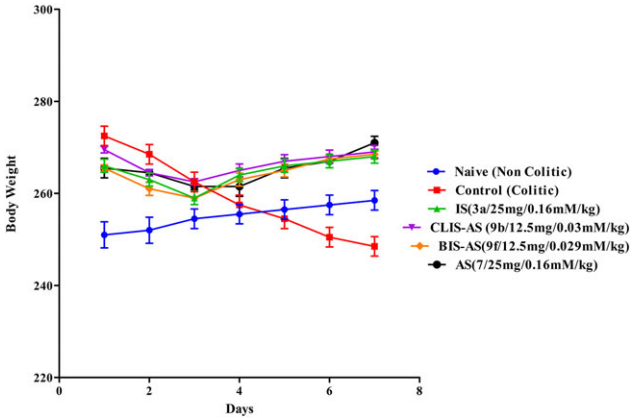


Figure 3. Body weight alterations in naïve (non-colitic), control (colitic), IS (**3a**, 0.16 mM/25 mg/kg), CLIS-AS (**9b**, 0.03 mM/12.5 mg/kg), BRIS-AS (**9f**, 0.029 mM/12.5 mg/kg), and AS (**7**, 0.16 mM/25 mg/kg). [Color figure can be viewed at wileyonlinelibrary.com]

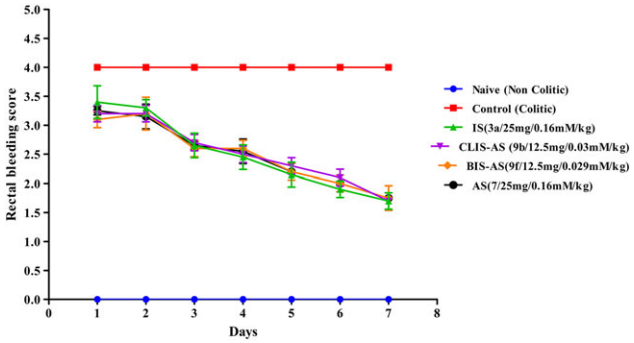


Figure 4. Rectal bleeding score in naïve (non-colitic), control (colitic), IS (**3a**, 0.16 mM/25 mg/kg), CLIS-AS (**9b**, 0.03 mM/12.5 mg/kg), BRIS-AS (**9f**, 0.029 mM/12.5 mg/kg), and AS (**7**, 0.16 mM/25 mg/kg). [Color figure can be viewed at wileyonlinelibrary.com]

(1.53 g, 0.01 mol) was dissolved in a mixture of 25 mL of glacial acetic acid and 25 mL of saturated solution of sodium acetate. To this, chloroacetyl chloride (1.5 mL, 0.013 mol) was added dropwise to avoid the vigorous reaction. After half an hour, the product was filtered with suction, washed several times with cold water, and then infrared dried. The product was crystallized from methanol [27].

General procedure for the preparation of isatin–mesalamine hybrids (9a–9g). Compounds **3a–3d**, **4**, **5** and **6** (0.0028 mol) were taken up in 30 mL anhydrous DMF and ice cooled with stirring. The reaction mixture was turned into dark purple color after the addition of solid K_2CO_3 (1 g, 0.0072 mol) in one portion that is raised to room temperature and stirred further for 1 h. The weighed amounts of N-acylated mesalamine (**8**) (1 g, 0.0028 mol) and KI (0.5 g, 0.006 mol) were added, and the reaction mixture was stirred at 80°C for 2–24 h until the reaction was over, which was confirmed using TLC. The reaction mixture was poured into six times of its volume to water, and then the diluted hydrochloric acid was added until the pH value is between three and four and stirred for a further 10 min. The crude final product was filtered with suction, washed several times with cold water, and then infrared dried. The product was crystallized from methanol [28]. The yield, melting point, and spectral data of each compound are given below.

1H-Indole-2,3-dione (3a). Solid, yield: 60%; mp 193–195°C; IR (KBr, t cm^{-1}): 3195 (NH of isatin NH), 1730 (C=O of isatin), 1696 (–NH–CO); ^{1}H NMR (300 MHz, δ ppm, DMSO-d_6): 6.95–8.03 (m, 4H, Ar–H), 10.20 (s, 1H, –NH); MS m/z (+ve): 148.1 (M^+). Elemental *Anal.* Calcd (%) for $C_8H_5NO_2$: C (65.31), H (3.43), N (9.52). Found: C (65.11), H (3.20), N (9.71).

5-Chloro-1H-indole-2,3-dione (3b). Solid, yield: 68%; mp 255–258°C; IR (KBr, t cm^{-1}): 3346 (NH of isatin), 1710 (C=O of isatin), 1683 (–NH–CO); ^{1}H NMR (300 MHz, δ ppm, DMSO-d_6): 7.50–7.53 (dd, 1H, Ar–H), 7.75–7.78 (dd, 1H, Ar–H), 7.80 (s, 1H, Ar–H), 8.94 (s, 1H, –NH); MS *m/z* (+ve): 181.9 (M^+), 183.9 (M + 2). Elemental *Anal.* Calcd (%) for $C_8H_4ClNO_2$: C (52.92), H (2.22), N (7.71). Found: C (52.88), H (1.96), N (7.82).

5-Fluoro-1H-indole-2,3-dione (3c). Solid, yield: 56%; mp 224–227°C; IR (KBr, t cm^{-1}): 3338 (NH of isatin), 1740 (C=O of isatin), 1672 (–NH–CO), 1227 (C–F); ^{1}H NMR (300 MHz, δ ppm, DMSO-d_6): 6.95–6.99 (dd, 1H, Ar–H), 7.50–7.54 (dd, 1H, Ar–H), 7.67–7.70 (dd, 1H, Ar–H), 11.00 (s, 1H, –NH); MS *m/z* (+ve): 166.1 (M^+). Elemental *Anal.* Calcd (%) for $C_8H_4FNO_2$: C (58.19), H (2.44), N (8.48). Found: C (58.10), H (2.38), N (8.69).

5-Iodo-1H-indole-2,3-dione (3d). Solid, yield: 40%; mp 276–280°C; IR (KBr, t cm^{-1}): 3216 (NH of –NH–CO), 1744 (C=O of isatin), 1658 (–NH–CO); ^{1}H NMR (300 MHz, δ ppm, DMSO-d_6): 6.91–6.93 (dd, 1H, Ar–H), 7.51–7.54 (dd, 1H, Ar–H), 8.64–8.70 (dd, 1H, Ar–H), 8.86 (s, 1H, –NH); MS *m/z* (+ve): 273.9 (M^+). Elemental *Anal.* Calcd (%) for $C_8H_4INO_2$: C (35.19), H (1.48), N (5.13). Found: C (35.12), H (1.41), N (5.20).

5-Nitro-1H-indole-2,3-dione (4). Solid, yield: 50%; mp 251°C; IR (KBr, t cm^{-1}): 3238 (NH of –NH–CO), 1730 (C=O of isatin), 1663 (–NH–CO), 1500–1480 and 1350–1318 ($–NO_2$); ^{1}H NMR (300 MHz, δ ppm, DMSO-d_6): 7.98–8.72 (m, 3H, Ar–H), 10.01 (s, 1H, –NH); MS *m/z* (+ve): 193.02 (M^+). Elemental *Anal.* Calcd (%) for $C_8H_4N_2O_4$: C (50.10), H (2.10), N (14.58). Found: C (49.99), H (1.86), N (14.92).

5-Bromo-1H-indole-2,3-dione (5). Solid, yield: 42%; mp 247–250°C; IR (KBr, t cm^{-1}): 3260 (NH of –NH–CO), 1728 (C=O of isatin), 1695 (–NH–CO), 678 (C–Br); ^{1}H NMR (300 MHz, δ ppm, DMSO-d_6): 6.97–7.03 (dd, 1H, Ar–H), 7.39–7.44 (dd, 1H, Ar–H), 7.76–7.80 (dd, 1H, Ar–H), 10.00 (s, 1H, –NH); MS *m/z* (+ve): 225.9 (M^+), 227.9 (M + 2). Elemental *Anal.* Calcd (%) for

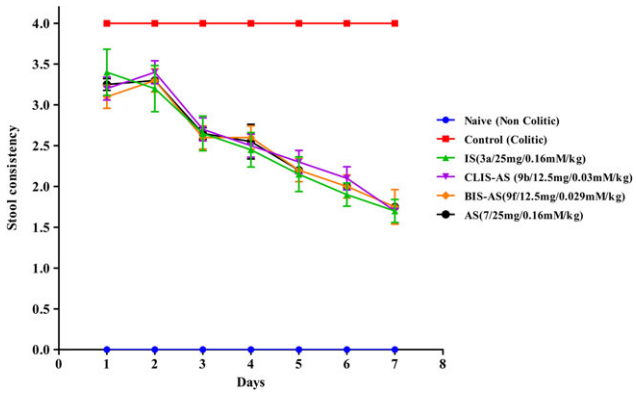
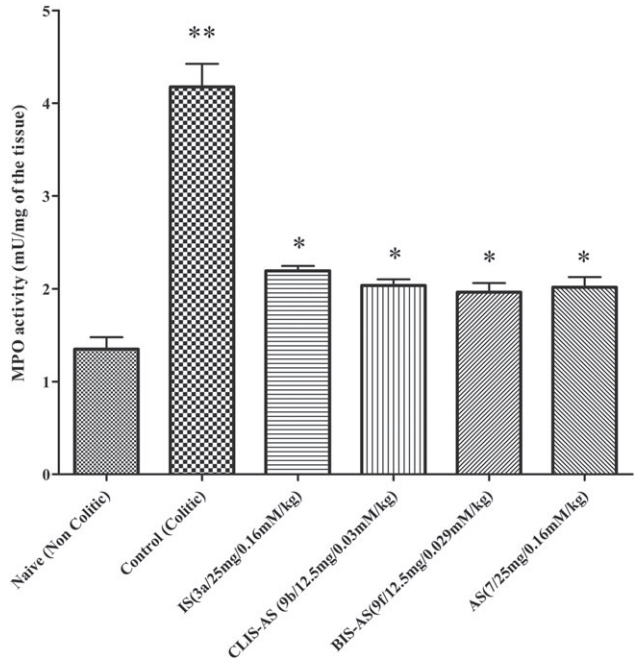
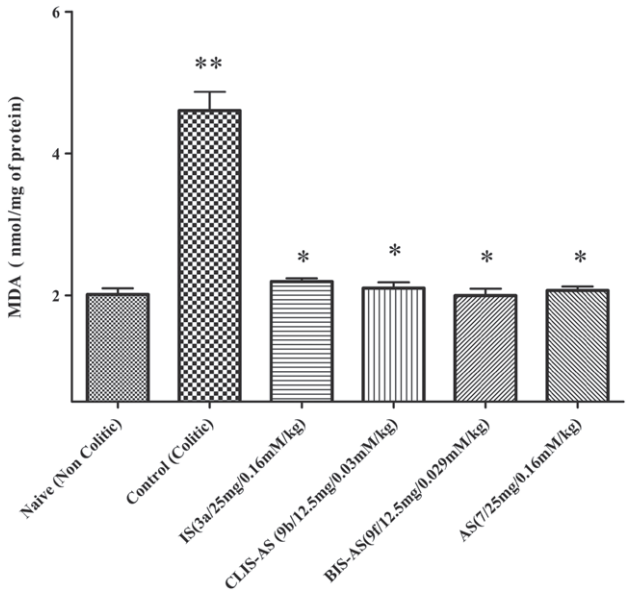


Figure 5. Stool consistency in naïve (non-colitic), control (colitic), IS (**3a**, 0.16 mM/25 mg/kg), CLIS-AS (**9b**, 0.03 mM/12.5 mg/kg), BRIS-AS (**9f**, 0.029 mM/12.5 mg/kg), and AS (**7**, 0.16 mM/25 mg/kg). [Color figure can be viewed at wileyonlinelibrary.com]



*p < 0.05 when compared with control (colitic) group.
**p < 0.01 when colitis group compared with naïve group.

Figure 6. Colonic MPO activity (mU/mg). MPO, myeloperoxidase.



*p < 0.05 when compared with control (colitic) group.
**p < 0.01 when colitis group compared with naïve group.

Figure 7. Colonic MDA activity (nmol/mg). MDA, malondialdehyde.

$C_8H_4BrNO_2$: C (42.51), H (1.78), N (6.20). Found: C (42.30), H (1.70), N (6.50).

5,7-Dibromo-1H-indole-2,3-dione (6). Solid, yield: 36.2%; mp 252–255°C; IR (KBr, t cm^{-1}): 3386 (NH of –NH–CO), 1725 (C=O of isatin), 1698 (–NH–CO); ^{1}H NMR (300 MHz, δ ppm, DMSO-d_6): 7.80 (s, 1H, Ar–H), 7.90 (s, 1H, Ar–H), 9.81 (s, 1H, –NH); MS *m/z* (+ve): 304.8 (M^+), 302.8 (M − 2), 306.8 (M + 2). Elemental *Anal*. Calcd (%) for $C_8H_3Br_2NO_2$: C (31.51), H (0.99), N (4.59). Found: C (31.42), H (0.90), N (4.73).

5-[2-(2,3-Dioxo-2,3-dihydro-indol-1-yl)-acetylamino]-2-hydroxy-benzoic acid (9a). Solid, YIELD: 40%; mp 218–220°C; IR (KBr, t cm^{-1}): 3490 (OH of –COOH), 3250 (NH of –NH–CO), 1725 (C=O of –COOH), 1700 (C=O of isatin), 1650 (–NH–CO); ^{1}H NMR (300 MHz, δ ppm, DMSO-d_6): 3.80 (s, 2H, H2C–CO), 6.75–7.02 (dd, 1H, Ar–H), 7.05–7.30 (ddd, 1H, Ar–H), 7.33–7.55 (dd, 1H, Ar–H), 7.58–7.63 (ddd, 1H, Ar–H), 7.66–7.79 (ddd, 2H, Ar–H), 8.25–8.30 (dd, 1H, Ar–H), 9.32 (s, 1H, Ar–OH), 9.49 (s, 1H, –NH), 11.90 (bs, 1H, –COOH–); ^{13}C NMR (300 MHz, δ ppm, DMSO-d_6): 53.0, 115.4, 116.0, 118.0, 121.3, 122.1, 124.9, 128.3, 130.4, 131.0, 135.0, 148.2, 158.0, 160.3, 168.6, 169.6, 184.2; MS *m/z* (+ve): 341.1 (M^+), 342.1 (M + 1). Elemental *Anal*. Calcd (%) for $C_{17}H_{12}N_2O_6$: C (60.00), H (3.55), N (8.23), O (28.21). Found: C (59.62), H (3.43), N (8.58), O (28.86).

5-[2-(5-Chloro-2,3-dioxo-2,3-dihydro-indol-1-yl)-acetylamino]-2-hydroxy-benzoic acid (9b). Solid, yield: 50%; mp 193–195°C; IR (KBr, t cm^{-1}): 3310 (NH of –NH–CO), 1722 (C=O of –COOH), 1705 (C=O of isatin), 1675 (–NH–CO), 558 (–C–Cl); ^{1}H NMR (300 MHz, δ ppm, DMSO-d_6): 3.88 (s, 2H, H2C–CO), 6.90–7.03 (dd, 2H, Ar–H), 7.10–7.41 (dd, 1H, Ar–H), 7.46–7.55 (dd, 1H, Ar–H), 8.14–8.25 (dd, 1H, Ar–H), 8.29–8.38 (dd, 1H, Ar–H), 9.22 (s, 1H, Ar–OH), 9.86 (s, 1H, –NH), 11.06 (bs, 1H, –COOH–); ^{13}C NMR (300 MHz, δ ppm, DMSO-d_6: 52.9, 115.0, 116.3, 119.2, 121.0, 123.5, 128.2, 130.4, 131.2, 135.4, 146.1, 158.4, 160.4, 168.5, 169.3,

184.3; MS *m/z* (+ve): 373.3 (M^+), 315.1 (M + 2). Elemental *Anal*. Calcd (%) for $C_{17}H_{11}ClN_2O_6$: C (54.49), H (2.96), N (7.48), O (25.62). Found: C (54.78), H (2.89), N (7.67), O (25.873).

5-[2-(5-Fluoro-2,3-dioxo-2,3-dihydro-indol-1-yl)-acetylamino]-2-hydroxy-benzoic acid (9c). Solid, yield: 36.5%; mp 186–188°C; IR (KBr, t cm^{-1}): 3520 (OH of –COOH), 3297 (–NH of –NH–C=O), 1710 (C=O of isatin), 1668 (–NH–CO), 1260 (–C–F); ^{1}H NMR (300 MHz, δ ppm, DMSO-d_6): 3.93 (s, 2H, H2C–CO), 6.85–7.05 (dd, 1H, Ar–H), 7.08–7.19 (dd, 1H, Ar–H), 7.26–7.35 (dd, 1H, Ar–H), 7.38–7.47 (dd, 1H, Ar–H), 7.84–7.91 (dd, 1H, Ar–H), 8.19–8.26 (dd, 1H, Ar–H), 9.34 (s, 1H, Ar–OH), 9.94 (s, 1H, –NH), 11.68 (bs, 1H, –COOH); ^{13}C NMR (300 MHz, δ ppm, DMSO-d_6): 53.1, 114.9, 115.4, 115.9, 118.6, 121.2, 121.6, 124.0, 128.2, 1308, 143.4, 158.0, 159.1, 160.2, 168.1, 169.2, 183.7; MS *m/z* (−ve): 357.2, (M^+), 355.2 (M + 1). Elemental *Anal*. Calcd (%) for $C_{17}H_{11}FN_2O_6$: C (56.99), H (3.09), N (7.82), O (26.79). Found: C (57.08), H (3.21), N (7.28), O (26.95).

2-Hydroxy-5-[2-(5-iodo-2,3-dioxo-2,3-dihydro-indol-1-yl)-acetylamino]-benzoic acid (9d). Solid, yield: 36%; mp 248–250°C; IR (KBr, t cm^{-1}): 3500 (OH of –COOH), 1712 (C=O of –COOH), 1684 (C=O of isatin), 1660 (–NH–CO), 572 (–C–I); ^{1}H NMR (300 MHz, δ ppm, DMSO-d_6): 3.92 (s, 2H, H2C–CO), 6.90–6.99 (dd, 1H, Ar–H), 7.36–7.43 (dd, 1H, Ar–H), 7.47–7.56 (dd, 1H, Ar–H), 7.59–7.67 (dd, 1H, Ar–H), 7.70–7.78 (dd, 1H–Ar–H), 8.23–8.30 (dd, 1H, Ar–H), 9.44 (s, 1H, Ar–OH), 10.04 (s, 1H, –NH), 12.2 (bs, 1H, –COOH; ^{13}C NMR (300 MHz, δ ppm, DMSO-d_6): 53.5, 90.8, 115.8, 116.3, 119.3, 121.2, 124.0, 128.2, 130.8, 138.0, 144.0, 147.1, 158.0, 160.4, 168.8, 169.2, 184.7; MS *m/z* (−ve): 464.9, (M^+), 465.9 (M + 1). Elemental *Anal*. Calcd (%) for $C_{17}H_{11}IN_2O_6$: C (43.80), H (2.38), N (6.01), O (20.59). Found: C (43.55), H (2.40), N (6.86), O (21.00).

2-Hydroxy-5-[2-(5-nitro-2,3-dioxo-2,3-dihydro-indol-1-yl)-acetylamino]-benzoic acid (9e). Solid, yield: 26%; mp 255–257°C; IR (KBr, t cm^{-1}): 3500 (OH of –COOH), 3440 (NH of –NH–CO), 1740 (C=O of –COOH), 1703 (C=O of isatin), 1658 (–NH–CO), 1550–1483 and 1353–1322 (–NO_2); ^{1}H NMR (300 MHz, δ ppm, DMSO-d_6): 3.86 (s, 2H, –CH2), 6.90–7.02 (dd, 1H, Ar–H), 7.33–7.42 (dd, 1H, Ar–H), 7.48–7.56 (dd, 1H, Ar–H), 8.19–8.26 (dd, 1H, Ar–H), 8.39–8.48 (dd, 1H, Ar–H), 8.66–8.73 (dd, 1H, Ar–H), 9.21 (OH of Ar–OH), 9.83 (s, 1H, –NH), 12.05 (bs, 1H, –COOH); ^{13}C NMR (300 MHz, δ ppm, DMSO-d_6): 53.8, 115.4, 116.0, 119.1, 121.3, 123.0, 125.0, 127.1, 128.3, 131.0, 145.0, 154.2, 158.0, 160.3, 168.6, 169.0, 183.3; MS *m/z* (−ve): 384.1 (M^+), 385.1 (M + 1). Elemental *Anal*. Calcd (%) for $C_{17}H_{11}N_3O_8$: C (53.00), H (2.88), N (10.91), O (33.22). Found: C (53.03), H (2.90), N (10.86), O (33.00).

5-[2-(5-Bromo-2,3-dioxo-2,3-dihydro-indol-1-yl)-acetylamino]-2-hydroxy-benzoic acid (9f). Solid, yield: 34.3%; mp 210–212°C; IR (KBr, t cm^{-1}): 3506 (OH of –COOH), 3430 (NH of –NH–CO), 1748 (C=O of –COOH), 1720 (C=O of isatin), 1667 (–NH–CO), 655 (C–Br); ^{1}H NMR (300 MHz, δ ppm, DMSO-d_6): 3.93 (s, 2H, H2C–C=O), 6.93–7.04 (dd, 1H, Ar–H), 7.19–7.27 (dd, 1H, Ar–H), 7.30–7.38 (dd, 1H, Ar–H), 7.42–7.49 (dd, 1H, Ar–H), 7.78–7.85 (dd, 1H, Ar–H), 7.98–8.26 (dd, 1H, Ar–H), 9.32 (bs, 1H, –OH), 9.89 (s, 1H, –NH), 11.16 (bs, 1H, –COOH); ^{13}C NMR (300 MHz, δ ppm, DMSO-d_6): 53.8, 115.4, 116.0, 119.1, 121.3, 123.0, 125.0, 127.1, 128.3, 131.0, 145.0, 154.2, 158.0, 160.3, 168.6, 169.0, 183.3; MS *m/z* (−ve): 401.3 (M^+), 403.3 (M + 2). Elemental *Anal*. Calcd (%) for $C_{17}H_{11}BrN_2O_6$: C (48.71), H (2.64), N (6.68), O (22.90). Found: C (48.64), H (2.58), N (6.73), O (22.85).

5-[2-(5,7-Dibromo-2,3-dioxo-2,3-dihydro-indol-1-yl)-acetylamino]-2-hydroxy-benzoic acid (9g). Solid, yield: 36.2%; mp 225–227°C; IR (KBr, t cm^{-1}): 3490 (OH of –COOH), 3275 (NH of –NH–CO), 1738 (C=O of –COOH), 1700 (C=O of isatin), 1664 (–NH–CO), 676 (C–Br); ^{1}H NMR (300 MHz, δ ppm, DMSO-d_6): 3.86 (s, 2H, H2C–C=O), 6.89–7.04 (dd, 1H, Ar–H), 7.35–7.43 (dd, 1H, Ar–H), 7.59–7.65 (d, 1H, Ar–H), 8.18–8.25 (dd, 1H, Ar–H), 8.28–8.33 (d, 1H, Ar–H), 9.37 (bs, 1H, –OH), 9.90 (s, 1H, –NH), 10.89 (bs, 1H, –COOH); ^{13}C NMR (300 MHz, δ ppm, DMSO-d_6: 53.1, 115.8, 116.4, 121.4, 125.3, 128.6, 131.0, 132.8, 140.3, 157.2, 160.4, 168.8, 184.8; MS *m/z* (−ve): 604.9 (M^+), 606.9 (M + 2), 602.9(M − 2). Elemental *Anal*. Calcd (%) for $C_{17}H_{10}Br_2N_2O_6$: C (40.99), H (2.02), N (5.62), O (19.27). Found: C (40.85), H (1.98), N (5.80), O (19.20).

BIOLOGICAL EVALUATION

DPPH free radical scavenging activity. The hydrogen atom donating ability of the different test compounds (**3a**–**3d**, **4**, **5**, **6**, and **9a**–**9g**) at various micromolar concentrations (200, 400, 600, 800, 1000 μM) in 9.5 mL of methanol, and 0.5 mL of DMSO was determined by the decolorization of 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol. According to the method reported by Blois [29], 0.2 mL of the test compound was added to 3 mL of 0.1 mM DPPH solution, vertexed for few minutes, and absorbance was read at 517 nm after 30 min. The decrease in absorption was correlated with the percent inhibition of samples. The percentage of inhibition was calculated by the following formula:

$$\%\ Inhibition\ of\ DPPH = [A_0 - A_s]/A_0 \times 100.$$

where A_0 is the absorbance of control and A_s is the absorbance of sample. Standard drug is Ascorbic acid.

Table 2

Experimental design for the evaluation of protective action of isatin–mesalamine conjugates (**9b**, **9f**), isatin (**3a**), and mesalamine (**7**) against acetic acid (AA)-induced colitis in rat model.

Groups	2 mL of normal saline *via* intra-colonic instillation (naïve)	AA-induced colitis (2 mL of 3% AA, intra-colonic) (control)	**3a** (0.16 mM/ 25 mg/kg) p.o. for 7 days	**9b** (0.030 mM/ 12.5 mg/kg) p.o. for 7 days	**9f** (0.029 mM/ 12.5 mg/kg) p.o. for 7 days	**7** (0.16 mM/ 25 mg/kg) p.o. for 7 days
I	+	–	–	–	–	–
II	–	+	–	–	–	–
III	–	+	+	–	–	–
IV	–	+	–	+	–	–
V	–	+	–	–	+	–
VI	–	+	–	–	–	+

Experimental design. Rats were randomized into groups, each consisting of six animals as shown in Table 2.

Isatin was dissolved in saline while 5-chloroisatin–mesalamine conjugate, 5-bromoisatin–mesalamine conjugate, and mesalamine were suspended in 0.5% carboxymethyl cellulose. The test compounds were administered to the rats once daily for 7 days as per the aforementioned protocol.

The effect of 0.5% carboxymethyl cellulose on the severity of acetic acid-induced ulcerative colitis was not examined in the present study as it has no effect on the intended biological action [30].

Induction of experimental colitis. Colitis was induced according to the method previously described by Millar *et al*. [17]. Briefly, animals received the treatment as per the specifications mentioned in Table 2 daily in the morning for 7 days. Test compounds treatment was started immediately after the intra-colonic administration of 2 mL of 3% acetic acid on day zero by using medical-grade polyurethane canal (external diameter 2 mm) that was inserted into the anus, and the tip was advanced to 8 cm proximal to the anus verge. The rats were maintained in the vertical position for 30 s in order to prevent the leakage of administered acetic acid from anus. The naïve group of animals received only 2 mL of normal saline solution. During the treatment period, animals were observed for the variations in body weight, rectal bleeding, and stool consistency. After the treatment period is over, on the 8th day, all the rats were sacrificed by decapitation. Colon was isolated and cut open to expose inner surface and washed thoroughly with normal saline and scanned for the morphological analysis. The remaining portions of colonic specimens were stored at −80°C until assayed for biochemical studies.

Assessment of colitis. ***Body weight, stool consistency, and rectal bleeding [31].*** The body weight of all the animals were measured daily from day 2 to until day 8. The stool consistency and rectal bleeding of all the animals was quantified on a 0–4 scale as shown in Table 3.

Macroscopic scoring. For each animal, the distal 10 cm portion of the colon was removed and cut longitudinally and slightly cleaned in physiological saline to remove fecal residues. Macroscopic inflammation scores were assigned based on clinical features of the colon using an arbitrary scale ranging from 0 to 4 as sorted in Table 4 [17].

Table 3

Stool consistency and rectal bleeding score description.

Score	Stool consistency	Rectal bleeding
0	Well-formed pellets	No blood
2	Pasty and semi-solid stool that did not stick to the anus	For positive finding
4	Liquid stools that sticks to the anus	Gross bleeding

Biochemical assays. Samples from the colon were stored immediately at −80°C until analysis. Tissue samples were homogenized in 10 mmol Tris–HCl buffer (pH 7.1), and the homogenate was used for the measurement of myeloperoxidase (MPO) and lipid peroxidation (MDA) enzymes.

Measurement of colonic lipid peroxides concentration. Thiobarbituric acid assay was used to measure the amount of colonic lipid peroxides as previously described by Ohkawa *et al*. [32]. Briefly, it consists of two steps: in the first step, 2.0 mL of freshly prepared 10% (w/v) TCA (trichloro acetic acid) was mixed thoroughly with 2.0 mL of the tissue homogenate, centrifuged, and kept in an ice bath for 15 min. In the second step, 2.0 mL of the freshly prepared thiobarbituric acid was mixed with 2.0 mL of

Table 4

Macroscopic scoring scale description.

Macroscopic scoring	Description
0	No macroscopic changes
1	Mucosal erythema only
2	Mild mucosal edema, slight bleeding, or small erosions
3	Moderate edema, slight bleeding ulcers, or erosions
4	Severe ulceration, edema, and tissue necrosis

clear supernatant solution from the first step, heated in a boiling water bath for 10 min and immediately cooled in an ice bath for 5 min. The color intensity was measured at 532 nm against reagent blank. MDA concentration was determined from standard graph. The values were expressed in nmol of MDA/mg protein.

Assessment of colonic myeloperoxidase activity. Myeloperoxidase activity was assessed according to the method described by Krawisz *et al*. [33]. Briefly, 50 mM of potassium phosphate buffer was prepared as a stock solution to dissolve 5% (w/v) DTAB (hexadecyltrimethylammonium bromide) and labeled it as reagent A. O-dianisidine dihydrochloride and 0.0005% hydrogen peroxide were also dissolved in the aforementioned buffer and named it as reagent B; 100 mg of colon mucosal scrapings were homogenized in reagent A solution and sonicated in ice bath for 10 s. The homogenates in reagent A solution were freeze–thawed thrice and centrifuged for 15 min at 20,000× *g*. The level of MPO activity was measured spectrophotometrically by mixing the 0.1 mL of the reagent A solution with 2.9 mL of the reagent B solution. The change in absorbance at 460 nm was measured for 5 min using a Shimadzu spectrophotometer. MPO activity was measured by using the following formula:

$$MPO\ activity\left(\frac{mU}{mg}\right) = 1000\times\left(\frac{X}{Weight\ of\ tissue\ taken\ (\mathrm{mg})}\right),$$

where $X = 10\times\frac{Change\ in\ absorption\ per\ minute}{Volume\ of\ supernant\ taken\ in\ final\ reaction}$.

Statistical analysis. The data were analyzed by using one-way ANOVA followed by Dunnet's test and two-way ANOVA (body weight, stool consistency, and rectal bleeding) followed by Bonferroni's test. All statistical tests were carried out with the help of GraphPad prism version 6 (trial version) software (Graph Pad Software, Inc. 7825, Fay Avenue, Suite 230, La Jolla, CA 92037, USA). All the values were expressed as mean ± standard error of the mean, and the criterion for statistical significance was considered to be $p < 0.05$.

CONCLUSION

In the present study, molecular hybridization technique was used to synthesize the isatin–mesalamine hybrids (**9a**–**g**) by employing previously reported standard methods. All the synthesized compounds (**3a**–**d**, **4**, **5**, **6**, and **9a**–**g**) were evaluated by DPPH free radical scavenging method in order to explore the antioxidant potential of the synthesized compounds. Among the tested compounds, hybrids **9b** (IC_{50} = 368.6 ± 3.5 μM) and **9f** (IC_{50} = 335.1 ± 2.9 μM) showed better antioxidant activity than its parent compounds such as **3a** (IC_{50} = 556.8 ± 2.9 μM), **5** (IC_{50} = 511.9 ± 3.6 μM), and **7** (IC_{50} = 768.9 ± 2.7 μM). Ulcerative colitis was induced by intra-rectal instillation of acetic acid into male Wistar rats so as to evaluate the potential of the synthesized compounds with better antioxidant activity (**9b**, **9f**) in the treatment of ulcerative colitis. An increase in the concentration of colonic MPO and MDA enzymes confirmed the induction of colitis in rats. These colonic MPO and MDA enzymes concentrations were significantly decreased upon the oral administration of hybrids **9b** and **9f** to colitic rat groups for 7 days. This shows that hybrids **9b** and **9f** are effective in ameliorating the ulcerative colitis than their parent moieties, mainly by reducing the reactive oxygen species as indicated by the decrease in the concentration of colonic MDA and MPO enzyme levels. Thus, it can be concluded that molecular hybridization offers wide opportunities with few limitations for the quest of the researchers to synthesize novel drugs with improved efficacy and safety over the existing drugs.

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