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Healing effects of *Aegle marmelos* (L.) Correa fruit extract on experimental colitis

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Graded doses of 50% ethanolic extract of dried fruit pulp of *Aegle marmelos* (AME) (100, 200 and 400 mg/kg) daily for 14 days in acetic acid (AA)-induced colitis in rats showed 200 mg/kg of AME as an optimal effective dose against AA-induced colonic damage score and weight. This dose (200 mg/kg; po) was further studied in AA-induced colitis for its effects on various physical (mucous/blood in stool, food and water intake and body weight changes), histology, antibacterial activity and biochemical parameters like free radicals (nitric oxide and lipid peroxidation), antioxidants (superoxide dismutase, catalase and reduced glutathione) and myeloperoxidase (acute-inflammatory marker) activities in rat colonic tissue. AME decreased colonic mucosal damage and inflammation (macroscopic and microscopic), mucous/bloody diarrhea, fecal frequency and increased body weight affected in AA-induced colitis. AME showed significant antibacterial activity and enhanced the antioxidants but decreased free radicals and myeloperoxidase activities thereby decreasing tissue damage and inflammation and thus, affording ulcer healing. The above effects of ***A. marmelos*** authenticated its use in indigenous system of Medicine.

Keywords: *Aegle marmelos*, Antioxidants, Colitis, Free radicals, Fruit pulp, Myeloperoxidase

Ulcerative colitis (UC) is a chronic inflammatory disorder involving the mucosa and sub-mucosa of the colon. It is largely a disease of the industrialized world, and is more common in urban areas and northern climates. It occurs at the rate of approximately five cases per 100,000 people[1]. The disease pattern is one of the remission and exacerbation and occurs most commonly among people between ages of 15-40 years. Though the exact etiology and pathophysiology is not known with certainty but genetic, immunological, reactive oxygen species (ROS) and environmental factors play a crucial role in the development of UC[2-4]. ROS leads to the formation of reactive peroxides and hydroxyl radicals which can cause lipid peroxidation by impairing cell membrane function and tissue damage.

Aegle marmelos (Rutaceae, Hindi: bael, AM), known as sripal or bilwa in Sanskrit and grows up to 18 meters tall and bears thorns and fragrant flowers. AM tree is indigenous to dry forests on hills and plains of India, Sri Lanka, Myanmar, Pakistan, Bangladesh, Nepal, Vietnam, Laos, Cambodia and Thailand. It has a woody-skinned, smooth fruit 5–15 cm in diameter. It has numerous seeds, which are densely covered with fibrous hairs and are embedded in a thick, gluey, aromatic pulp. AM has been advocated for the treatment of pain, fever, inflammation, respiratory disorders, cardiac disorders, dysentery and diarrhoea. Scientific studies have validated many of the ethnomedicinal uses such as anti-inflammatory, antipyretic and analgesic, anti-diarrheal, anti-diabetic, antifungal, anti-hyperlipidemic, antimicrobial, antibacterial and anti-parasitic, anti-cancer, anti-malaria, hepatoproctective and cardioprotective activities[5]. The fruit to contains many functional and bioactive compounds such as carotenoids, phenolics, alkaloids, coumarins, flavonoids, terpenoids, and other antioxidants. In addition, it also has many vitamins and minerals including vitamin C, vitamin A, thiamine, riboflavin, niacin, calcium, and phosphorus[6].

The use of complementary and alternative medicines, particularly of herbal therapies, for chronic illness such as diabetes, osteoporosis, cancer, and liver diseases is widespread and increasing[7-9], and inflammatory bowel disease (IBD) is not excluded from this trend[10]. In this context, considering the traditional uses of *Aegle marmelos*, the role of oxidative stress in the pathogenesis of IBD and the presence of a number of compounds with antioxidant

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and anti-inflammatory properties prompted us to investigate the healing effect of hydroalcoholic extract of dried fruit pulp of AM on AA-induced colitis in rats.

Materials and Methods

Animals—Inbred Charles-Foster (CF) strain albino rats (130-170 g) of either sex, were obtained from the Central animal house of the Institute. They were kept in the departmental animal house at 26 ± 2 °C, 44-56% RH and 10:14 hr L:D cycle for 1 week before and during the experiments. Animals were provided with standard rodent pellet diet (Pashu Aahar, Ramnagar, Varanasi) and water was given *ad libitum*. 'Principles of laboratory animal care' (NIH publication no. 82-23, revised 1985) guidelines were followed. Approval from the Institutional Animal Ethical Committee was taken prior to the experimental work (vide letter N0. Dean/2009-10/568 dated 10.08.2009). six animals were taken in each group. The animals were sacrificed with overdose of ether as and when required.

Plant material—Big sized, unripe, AM fruits were collected during months of November to March (Ayurvedic Gardens, Banaras Hindu University). The shell of the fruit was removed and the pulp was cut into small pieces and dried at room temperature and powdered and stored for further use. The plants and their parts were identified with the standard sample preserved in the Department of Dravyaguna, Institute of Medical Sciences, Varanasi.

Preparation of extract—Ethanolic extract (50%) of AM (AME) was prepared by adding 1 liter of ethanol in 200 g of dried fine powder of AM. The mixture was shaken at intervals and the extract so obtained was filtered after an interval of two days. The procedure was repeated twice at an interval of two days. The extract so obtained each time was mixed and later dried at 40 °C in incubator. The yield of the extracts was 12.7 %. Enough quantity of the extract was prepared fresh before use.

Chemicals—Drugs/chemicals used in the study were purchased from standard companies. Muller-Hinton agar and broth (Hi-Media, Mumbai, India), was used for antibacterial activity.

Treatment protocol—AME and standard UC protective drug, sulfasalazine (SS) were suspended in 0.5% carboxy methyl cellulose (CMC) in distilled water and were given in the volume of 1mL/100 g body weight. AME/SS was given orally once daily for 14 days after the induction of colitis with AA.

Experimental colitis and study of physical parameters—Colitis was produced by intracolonic administration of acetic acid (AA, 10%, 0.2 mL/rat)[11]. An initial dose response study was undertaken with AME (*100*, 200 and 400 mg/kg) and standard UC protective drug, SS (100 mg/kg) against AA-induced colonic mucosal damage score, weight and adhesions (Table 1). An optimal effective healing dose of AME (200 mg/kg) was then selected for further work on the number of stool frequency with or without fecal blood/mucus, food and water intake and body weight changes.

Pathology and histopathology of colon—Pathological changes (macroscopic) were seen by examination of 10 cm distal part of rat colon following the method of Morris *et al.*[12]. Colon was examined for severity and number of ulcers in terms of tissue damage score, thickening and adhesions (signs of inflammation). Histopathology of the colon was done in all the groups on 15th day to know the status of healing. A piece of colon was removed and fixed in 10 % buffered formalin and paraffin embedded. Sections (4-6 um thick) were stained with Hemotoxylin and Eosin stain for histological evaluation and examined under microscope at 100 X magnification.

Antibacterial activity—In a separate study *in vitro* antibacterial susceptibility test of AME was done using serial concentrations of 50, 100, 150 and 200 mg/mL following the approved standards of the National Committee for Clinical Laboratory Standards[13] against various intestinal pathogens i.e. *Escherichia coli* ATCC 25922, *Shigella boydii*, *Shigella sonnei* and *Shigella flexneri* obtained from the American Type Culture Collection (ATCC) and clinical strain preserved at Department of Microbiology, Institute of Medical Sciences, BHU, Varanasi, India following the disk diffusion method while, minimum inhibitory concentration (MIC) was performed by micro dilution method[14].

Biochemical parameters—Estimation of (i) protein[15], (ii) oxidative free radicals–lipid peroxidation (LPO)[16] and nitric oxide (NO)[17], (iii) antioxidants-superoxide dismutase (SOD)[18], catalase (CAT)[19] and reduced glutathione (GSH)[20] and (iv) inflammatory marker like myeloperoxidase (MPO)[21] were done in colonic tissue homogenates following the standard procedures. Briefly, LPO levels were estimated in terms of malondialdehyde (MDA) released during lipid peroxidation[16]. Nitrites and nitrates are formed as end products of reactive nitrogen products during

NO formation which are measured by using Griess reagent[17]. The assay of SOD is based on the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazon. One unit of enzyme activity is defined as the amount of enzyme that gave 50% inhibition of nitroblue tetrazolium reduction in one min[18]. CAT measurement was done based on the ability of catalase to oxidize H_2O_2. One unit (U) of catalase is the enzyme, which decomposes 1 mM of H_2O_2. per min at 25 °C[19]. GSH activity in the homogenate was estimated by the ability of GSH to reduce DTNB within 5 min of its addition against a reagent blank with no homogenate[20]. MPO activity was determined as an indicator of polymorphous nuclear leukocyte adhesion and accumulation in the colon. MPO activity was determined in the homogenate of the colonic tissue based on the principle of release of MPO enzyme. A unit of MPO activity is defined as that converting 1 μmol of H_2O_2 to water in 1 min at 25 °C[21].

Statistical analysis—The statistical analysis was carried out by using unpaired 't' test and one way analysis of variance followed by Dunnett's test for multiple comparisons. The values are represented as mean ± SE. P <0.05 was considered significant.

Results

Experimental colitis and study of physical parameters

Effects on colonic damage, inflammation and adhesions—Rats receiving 0.5% CMC orally through an orogastric tube, were given normal saline instead of AA in the colon intrarectally (NS, negative control group). They did not show any colonic mucosal damage and inflammation (0 ± 0) or adhesion (0/6) at 15th day of experiment while, the colonic weight (8 cm of proximal colon) expressed as mg/cm of colon was found to be 171.0 ± 4.8. The AA group received 0.5% CMC orally daily as above but was given AA in the colon intrarectally. AA treatment led to significant increase in colonic mucosal damage score (5.78 ± 0.22, P <0.001) and adhesions (4/6 adhesions, 66.7%) and increase in colonic weight from 171.0 to 261.2 mg/cm (52.7% increase) compared with NS group. AME when given in graded doses of 100, 200 and 400 mg/kg for 14 days after the induction of colitis with AA showed decrease in colonic damage score, colonic weight and adhesions compared with AA-colitis rat. Therefore, AME (200 mg/kg) showing percentage decrease in damage score >50% was selected for further studies on macroscopic and microscopic, physical (diarrhea, stool frequency and body weight) and biochemical (free radicals and antioxidant status) parameters when given for 14 days after the induction of colitis with AA (Table 1).

Effects on diarrhea, stool frequency and presence of blood or mucous—AA when instilled intra-rectally into the colon led to severe diarrhea in all the animals (100 %) which was prominent on day 2 and then decreased to 50 % of animals on day 4 and was associated with presence of blood till 4th day and mucous till day 10. Day 0 stool frequency varied in different groups of rats. AA colitis rats showed increased fecal frequency from day 2 onwards which gradually increased over day 4 and then started decreasing from day 6 onwards and at day 14 it was 140.4% compared with day 0 frequency.

Table 1—Effects of graded doses of 50% ethanolic extract ofdried fruit pulp of *A. marmelos* (AME) on acetic acid (AA)-induced rat colonic mucosal damage score, weight and adhesions
[Values are mean ± SE of 6 rats in each group]

Oral treatment (mg/kg, od x 14 days)	Damage score		Colonic weight	Adhesions
	(0-10)	(PDDS)	(mg/cm)	(%)
NS (0.5% CMC)	0.00	—	171.0 ± 4.80	0.00
AA (0.5% CMC)	5.78 ± 0.22*	0.0	261.2 ± 6.79*	66.7
AA + AME (100)	5.17 ± 0.17[a]	10.6	219.8 ± 6.73[c]	50.0
AA + AME (200)	1.83 ± 0.31[c]	68.3	183.5 ± 9.54[c]	33.3
AA + AME (400)	1.33 ± 0.21[c]	77.0	180.8 ± 13.4[c]	33.3
AA + SS (100)	1.33 ± 0.11[c]	77.0	159.1 ± 5.17[c]	16.7

Percentage decrease in damage scores (PDDS) = $(1-D_T/D_C) \times 100$, where D_T and D_C are damage score values of respective AME-treated and control AA group.
*P<0.001 compared to respective NS group (unpaired't' test); and [a] P<0.05, [c] P<0.001 compared to respective AA group (statistical analysis was done by one way analysis of variance followed by Dunnet 't's test for multiple comparisons).

Administration of AME in AA-colitis rats showed decrease in stool frequency from day 4 onwards which was comparable with SS (Fig. 1a).

Effects on body weight changes and food and water intakes—AA-induced colitis led to gradual decrease in body weight as observed from day 2 onwards till 14th day of study. Significant decrease on body weight was observed from 6th day onwards as compared with NS rats. Treatment with AME for 14 days reversed the decrease trend in body weight which was comparable with SS-treated rats (Fig. 1b). However, little or no change was observed on food and water intake between the AA-treated and AME and SS treated animals from 0 day to 14th day of study treatments.

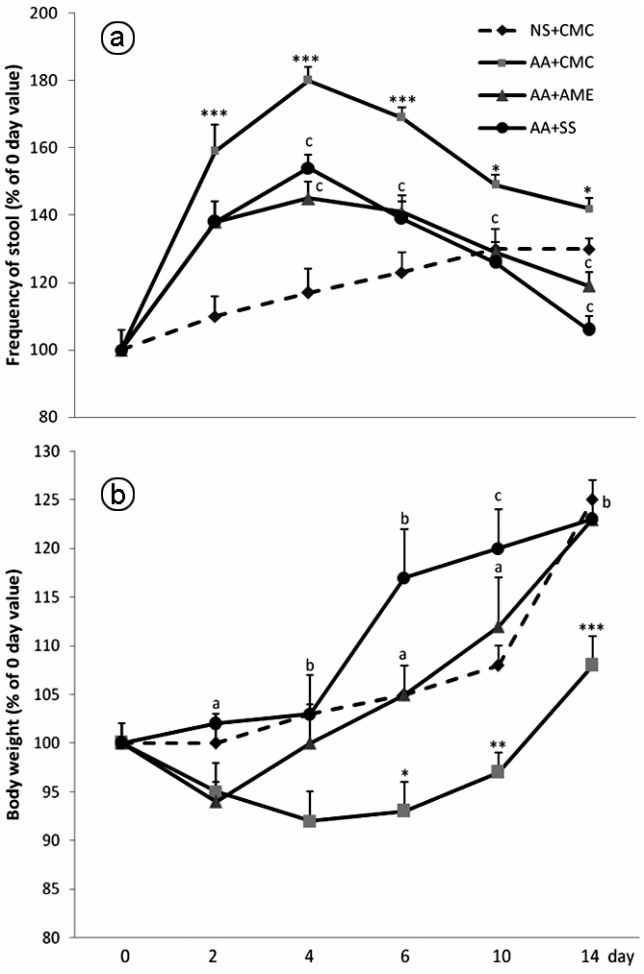

Fig. 1—Frequency of stool (a) and changes in body weight (b) in AME and SS treated AA-induced colitis rats. [*P* values: ^{*,a}<0.05, ^{**,b} <0.01, ^{***,c} <0.001; *, **, ***: compared to respective NS group (unpaired 't' test); $^{a,\ b,\ c}$ compared to respective AA group (one way analysis of variance followed by Dunnett's test for multiple comparisons)]

Pathology and histopathology of Colon:

Macroscopic study—Oral CMC-treated rats having normal structure of colon after NS enema is given in Fig. 2a. Fig. 2b showed the colon of oral CMC-treated rats after intracolonic AA administration having significant hydropsia, necrosis, erosion and ulceration. Figures 2c and d showed the colons with AA-induced colitis treated with AME and SS respectively. The severity of hydropsia, necrosis and ulceration were significantly reduced by AME and was comparable with that of SS-treated rats.

Microscopic study—Photomicrographs of colon shown in Fig. 3a-d provided convincing evidence for the protective effects of AME and SS on colitis induced by AA in the rats. Saline enema (NS)-treated colon of rats with oral CMC showed relatively normal and clear structure with intact mucosa and sub mucosa (Fig. 3a). AA colitis rats showed crypt destruction with severe cryptitis, ulceration with eroded mucosa, lymphoplasmacytic infiltrate and transmural inflammation (Fig. 3b). AA-induced colitis rats treated with AME showed regenerative mucosa

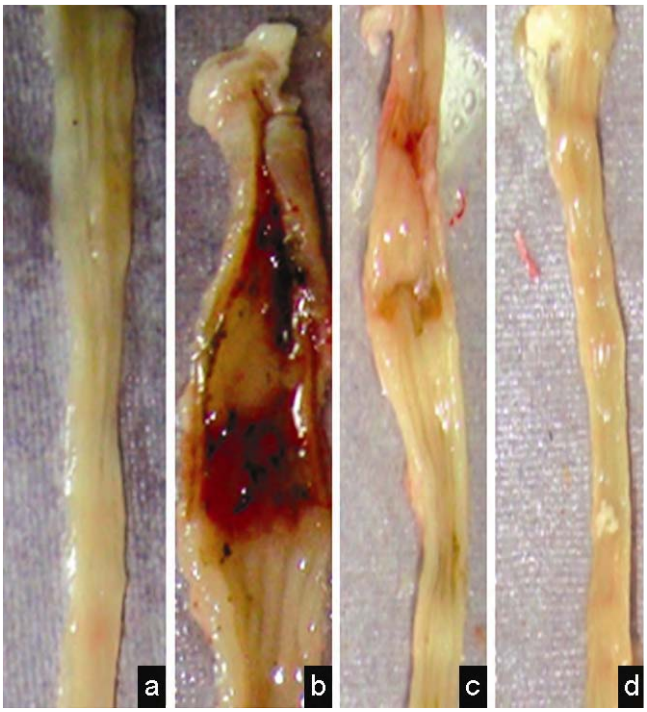

Fig. 2—Macroscopic changes in rat colonic mucosa. (a) NS enema treated colon of CMC-treated rat showing normal morphology, (b) colon of AA-induced colitis rat treated with CMC showing hydropsia, necrosis, erosion and ulceration, (c) colon of AA-induced colitis rat treated with AME (200 mg/kg) showing reduction in ulceration, erosion, necrosis and hydropsia, (d) colon of AA-induced colitis rat treated with SS (100 mg/kg) showing reduction in ulceration, erosion, necrosis and hydropsia.

with mild crypt distortion and mild lympho-plasmacytic infiltrate in the lamina propria with oedematous submucosa while, SS-treated rat showed intact mucosa with minimal lympho-plasmacytic infiltrate in the lamina propria (Fig. 3c and d).

Antibacterial activity

Antimicrobial susceptibility and MIC—In vitro antimicrobial test (Table 2) showed the susceptibility test against gram negative bacteria with AME showing antibacterial activity at 200 mg/mL against all the above gram negative intestinal bacteria. MIC value against the above intestinal microorganism ranged from 12.5-25.0 mg/mL, where the MIC value against *E. coli* and *S. boydii* was 12.5 mg/mL compared to 25.0 mg/ml against *S. sonnie* and *S. flexneri.*

Biochemical parameters

Effects on free radicals—AA enhanced both LPO and NO levels in the colonic mucosal incubates when expressed as nmol MDA/mg protein and nmol/mg protein wet tissue compared to NS rats. AME and SS showed reversal of levels of both LPO and NO near to the NS level (Table 3).

Effect on antioxidants—AA treated animals showed significant decrease in SOD, CAT and GSH levels in the colonic mucosal incubates when expressed as mU (SOD and CAT) or nmol (GSH) per mg protein compared to NS group. AME and SS treatments after AA-induction of colitis reversed the above changes in SOD, CAT and GSH levels near to NS group (Table 3).

Effect on myeloperoxidase—AA treated animals showed significant increase in MPO level in the

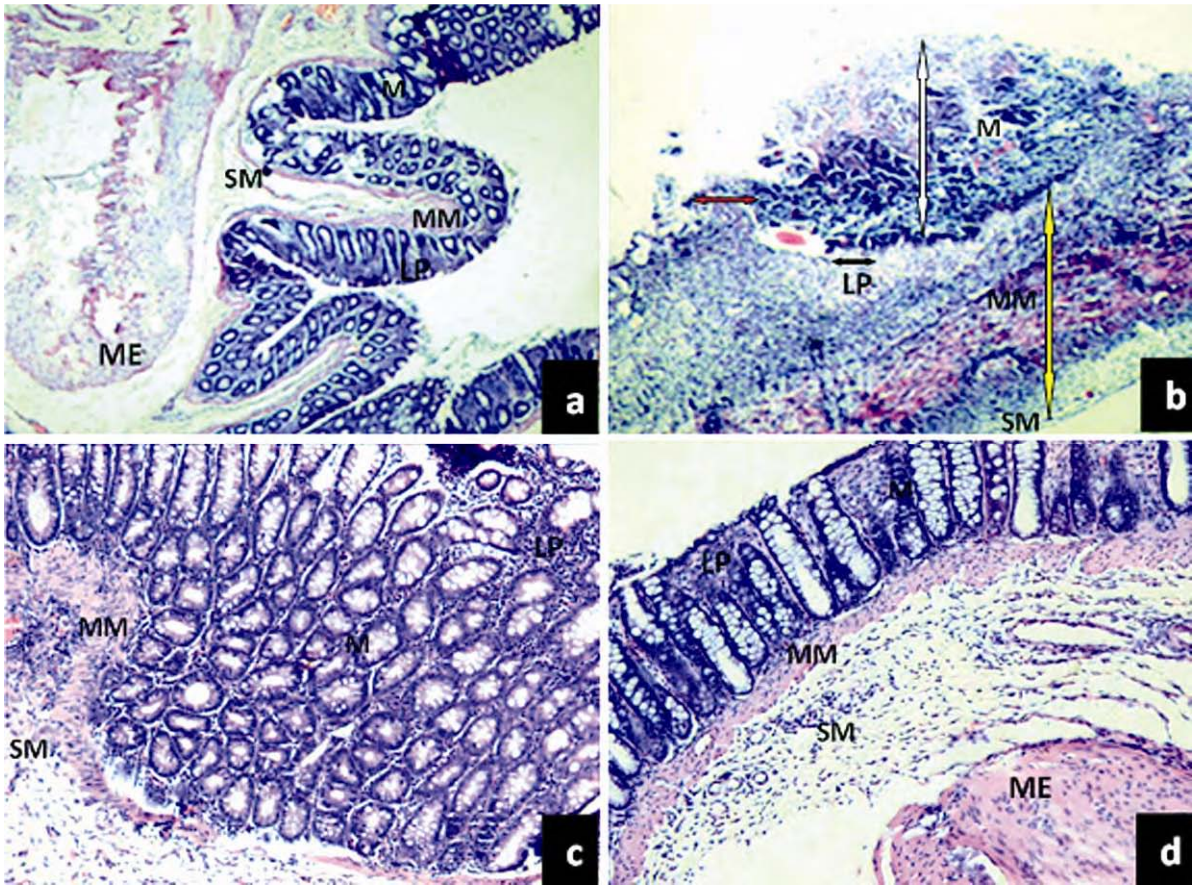

Fig. 3—Histolgical section of rat colon stained with H & E stain (x100). (a) saline enema (NS) treated colon of rats with oral CMC. The structure is relatively normal and clear with intact mucosa and sub mucosa. (b-d) colon of AA-induced colitis (b) treated with oral CMC showing ulcerated and eroded mucosa shown by white arrow, crypt destruction with severe cryptitis shown by brown arrow, lymphoplasmacytic infiltrate shown by black arrow and transmural inflammation (predominantly-lymphocytes and plasma cells) (c) treated with AA+AME showing regenerative mucosa with mild crypt distortion and mild lympho-plasmacytic infiltrate in the lamina propria with oedematous submucosa and (d) treated with AA+SS showing intact mucosa with minimal lymphoplasmacytic infiltrate in the lamina propria. [M: mucosa; SM: submucosa; MM: muscularis mucosa; LP: lamina propria; ME: muscularis externa]

Table 2—Antibacterial activity and minimum inhibitory concentration (MIC) of AME
[Values are mean ± SE of 3 experiments in each group]

Name of organism	AME antibacterial activity (zone of inhibition in mm)				MIC
	(50 mg/mL)	(100 mg/mL)	(150 mg/mL)	(200 mg/mL)	(mg/mL)
E. coli ATCC 25922	7.17 ± 0.12	8.37 ± 0.32	9.13 ± 0.26	10.1 ± 0.31	12.5
Shigella sonnie	6.23 ± 0.56	7.20 ± 0.55	8.30 ± 0.35	10.4 ± 0.33	25.0
S. boydii	7.13 ± 0.27	8.30 ± 0.31	8.60 ± 0.29	10.7 ± 0.54	12.5
S. flexneri	7.20 ± 0.32	7.30 ± 0.32	8.33 ± 0.33	10.8 ± 0.55	25.0

Table 3—Effect of AME and SS on AA-induced changes in free radicals (lipid peroxidation, LPO and nitric oxide, NO), antioxidants (superoxide dismutase, SOD; catalase, CAT and glutathione, GSH) and myeloperoxidase (MPO) in rat colonic mucosa
[Values are mean ± SE of 6 rats in each group]

Oral treatment (mg/kg, od x 14 days)	Free Radicals		Anti-oxidants			Myeloperoxidase
	LPO	NO	SOD	GSH	CAT	MPO
	(nmol/mg protein)		(mU/mg protein)	(nmol/mg protein)	(mU/mg protein)	(mU/mg protein)
NS (0.5% CMC)	6.26 ± 0.31	4.91 ± 0.61	161.8 ± 23.3	12.68 ± 0.66	2.49 ± 0.25	6.23 ± 1.18
AA (0.5% CMC)	10.98 ± 0.77*	9.95 ± 0.60*	24.8 ± 3.82*	7.18 ± 0.63*	0.96 ± 0.15*	74.0 ± 3.55*
AA + AME (200)	7.11 ± 0.61 b	4.66 ± 0.34 c	180.1 ± 29.8 c	10.22 ± 0.45 b	1.67 ± 0.11 b	15.6 ± 2.47 c
AA + SS (100)	5.11 ± 0.56 c	3.54 ± 0.30 c	294.4 ± 37.9 c	11.82 ± 1.13 b	2.36 ± 0.24c	8.04 ± 1.63 c

*P<0.001 compared to respective NS group (unpaired 't' test); and [b]P<0.01 and [c] P<0.001 compared to respective AA group (statistical analysis was done by one way analysis of variance followed by Dunnett's test for multiple comparisons).

colonic mucosal incubates when expressed as mU/mg protein compared to NS rats. AME and SS reversed the above changes in MPO level near to NS group (Table 3).

Discussion

Acetic-acid-induced colitis is an easily inducible model of IBD and the inflammatory phase bears some resemblance to acute human intestinal inflammation[22]. The present results with intra-colonic administration of acetic acid indicated significant hydropsia, necrosis, erosion and ulceration. Acetic acid treatment led to significant increase in colonic mucosal damage score and adhesions (indicative of necrosis and ulcerations) and increase in colonic weight (indicative of inflammation and hydropsia). Microscopic study of the colon indicated crypt destruction with severe cryptitis, ulceration with eroded mucosa, lymphoplasmacytic infiltrate and transmural inflammation. Rats showed increase in diarrhea with mucous and blood present in the early phase and increased fecal frequency in the later phase after induction of colitis with AA. This could be due to direct damaging effects of AA as well as alterations in epithelial function produced, either directly or indirectly by products released from activated mast cells[23]. Loss of body weight without any significant change in the food and water intake which could be due to alterations in the GIT absorptive functions. Treatment with AME reduced the severity and extension of damage both macroscopic as well as microscopic induced by AA. This was accompanied by increase in body weight, decrease in diarrhea, fewer incidences of adhesions and decreased lympho-plasmacytic infiltration. These above effects may be attributed to the anti-inflammatory, immunomodulatory and antiulcer properties of AM[24-26].

Experimental and clinical evidences suggest that the inflamed colon undergoes substantial oxidative stress by neutrophils-derived oxidants and MPO activity, both of which contribute markedly to tissue damage during chronic intestinal inflammation[27,28]. Measurement of MPO activity has been used as an indicator of neutrophils influx into inflamed gastrointestinal tissue[29]. The present study showed a significant increase in myeloperoxidase activity in the acetic acid group. This provides a quantitative measure of disease severity and a method of assessing drug efficacy in animal models of intestinal inflammation[30]. Such an infiltration might be regarded as a trigger of free radicals release which may exert toxic effects on fatty acid residues in membrane lipids. Increase in reactive oxygen species production and impaired antioxidant defense mechanisms are

postulated to be causative factors in inflammatory diseases. Therefore, elimination of ROS could be an important strategy in treatment of IBD and antioxidants have been postulated to hasten the process of healing by destroying the free radicals[31]. IBD have been found to be associated with an overproduction of nitric oxide (NO) by the inducible isoform of NO synthase (iNOS)[32]. The result studies on the antioxidants, SOD, CAT and GSH, free radicals, LPO and NO and myeloperoxidase, an inflammatory marker status revealed that AME possessed significant antioxidant activity reducing free radicals stress and decrease in colonic MPO which would help to prevent oxidative damage and promote the healing process.

AM fruit pulp contains important bioactive compounds such as carotenoids, phenolics, alkaloids, pectins, tannins, coumarins, flavonoids and terpenoids[33-36]. Extract of *A. marmelos* has been reported to possess immunomodulatory activity in experimental models of cellular and humoral immunity[37]. Flavonoids are most commonly known for their antioxidant activity. Flavonoids are known to reduce lipid peroxidation not only by preventing or slowing the onset of cell necrosis but also by improving vascularity.

Some studies have suggested the role of microbial content of the GI tract in the pathogenesis of IBD and it has been shown that the concentration of intestinal bacteria in IBD is higher than normal and increases progressively with disease severity[38]. The complex enteric immune system plays an important role in interactions between microbial antigens and immune-competent cells. AME exhibited considerable level of inhibition against the intestinal organisms compared to standard drug. This could be due to the presence of some compounds or groups in the extract with similar mechanism of action to that of standard drug used. The highest activity exhibited by AME at 200 mg/mL concentration was around 10 mm which may be considered as active dose[13]. The antibacterial results with AME corroborates with the findings of Maheshwari *et al.*[39] where they have studied the effects of ethanolic extract of dried fruit pulp of *A. marmelos* against various intestinal pathogens i.e. *Shigella boydii*, *S. sonnei* and *S. flexneri* and proposed that certain phytochemicals including phenols, tannins and flavonoids were effective against all. This could be contributory factors in helping healing of colitis induced by AA the present study also.

Greater expression of cytokines such as interleukin (IL)-1b, IL-6, IL-12, tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ) were demonstrated in patients with IBD[40, 41]. Destruction of the intestinal mucosa by intracolonic administration of AA may be associated with amplified production of TNF-α secretion which could lead to activation of further cytokine production by macrophages and in addition can act directly on intestinal epithelial cells, initiating the changes in epithelial permeability and ion transport that lead to mucosal inflammation and lesions[42]. Recent studies have shown that some herbal therapies improve IBD in human or animal models in this way. Thus, further work will be required for an in depth study to evaluate the effect of AME on above factors for coming to a definite conclusion of role of AM in ulcerative colitis for which it has been advocated in indigenous system of medicine. The results of present study with the 50 % ethanolic extract of dried fruit pulp of *Aegle marmelos* on various physical and biochemical parameters of colonic damage and inflammation induced by AA do indicate promising healing effects.

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