# Project Report

**"Study of protective and healing effects of *Aegle marmelos, Terminalia chebula* and *Azadirachta indica* on experimental models of ulcerative colitis in rats"**


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**Ministry of AYUSH, AYUSH Bhawan, B-Block, GPO Complex, INA, New Delhi-110023**

# FINAL REPORT

**(01.01. 2011 to 31.12. 2012)**

**1. Title of the Project:** Study of protective and healing effects of *Aegle marmelos, Terminalia chebula* and *Azadirachta indica* on experimental models of ulcerative colitis in rats.

**2. PI (name and address):** Prof. R.K. Goel, HOD, Department of Pharmacology, IMS, BHU, Varanasi-2210055

**3. Co-I (name and address):**

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**ii)** Co-I-2 : Prof. D. Dash**,** Dept. of Biochemistry, IMS, BHU, Varanasi-5

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**iv)** Co-I-4: Prof. Mohan Kumar, Dept. of Pathology, IMS, BHU, Varanasi-5

**4. Other Scientific Staff engaged in the study:** Mr. Manish Kumar Gautam, Research Associate

**5. Non-Scientific Staff engaged in the study:** Mr. Dinesh Kumar, Laboratory Attendant

**6. Implementing Institution and other collaborating Institutions:**
Institute of Medical Sciences, Banaras Hindu University

**7. Date of commencement:** 1st January, 2011

**8. Duration:** 3 years

**9. Date of completion: 31st December 2013**

10. **Objectives as approved:** To evaluate the ulcer protective effects of extracts of *A. marmelos*, *Terminalia* chebula and *Azadirachta indica* on experimental models of ulcerative colitis in rats.

11. **Deviation made from original objectives if any, while implementing the project and reasons thereof:** An initial dose-response study was done with 50% ethanolic extract of the dried fruit pulps of *A. marmelos and Terminalia chebula* and dried leaves of *Azadirachta indica* to find the optimal effective dose of the extracts and then a comparative study of same optimal doses of aqueous, ethanolic and 50% ethanolic extract of the above three plants was done which indicated better effects of 50% ethanolic extract of these plants on colonic damage score and weight and adhesions than either aqueous or 100% ethanolic extracts. Further protective (3 days before + 14 days treatment after colitis) and healing (14 days treatment after induction of colitis) effects with the optimal effective doses of the 50% ethanolic extract of all the plants studied, showed similar effects on colonic damage score and weight and adhesions so for detailed study on various physical and biochemical parameters the work was done only using 50% ethanolic extracts of these plants for healing study only. In our first report to the CCRAS, we reported an initial dose-response study with 50% ethanolic extract of the dried fruit pulps of *A. marmelos and Terminalia chebula* and dried leaves of *Azadirachta indica* against acetic acid-induced colitis in rats to find the optimal effective dose of the extracts and then a comparative study of same optimal doses of aqueous, ethanolic and 50% ethanolic extract of the above three plants was done against acetic acid (AA)-induced colitis in rats which indicated better effects of 50% ethanolic extract of these plants on colonic damage score and weight and adhesions than either only aqueous or 100% ethanolic extracts. Further protective (3 days before + 14 days treatment after colitis) and healing (14 days treatment after induction of colitis) effects with the optimal effective doses of the 50% ethanolic extract of all the plants studied, showed similar effects on colonic damage score and weight and adhesions so for detailed study on various physical and biochemical parameters the work was done only using 50% ethanolic extracts of these plants for healing study only both against AA/TNBS (Trinitrobenzene sulphonic acid)-induced colitis in rats.

12. **Experimental work giving full details of experimental set up, methods adopted, data collected supported by necessary tables, charts, diagrams and photographs:** Annexure I & II.

13. **Detailed analysis of results indicating contributions made towards increasing the state of knowledge in the subject**: The results of the present study do indicate the healing effects of 50% ethanol extract of dried fruit pulp of *A. marmelos* (AI) *and Terminalia chebula* (TC) and dried leaves of *Azadirachta indica* (AI) as mentioned in the indigenous system of medicine as well as in recent screening of these plants for their medicinal usage indicating their beneficial effects against inflammation, ulcer healing, diabetes etc. The present work indicated antioxidants activity in these plants and they were also found to decrease free radicals and myeloperoxidase, a pro-inflammatory marker indicating the protection afforded by the plants extract to tissue damage and inflammation. These effects have resulted in better colonic mucosal healing against AA- and TNBS-induced colonic damage as indicated by decrease in tissue adhesions, diarrhea and enhanced body weight and better healing.

14. **Conclusions summarizing the achievements and indication of scope for future work:** The results of present study with the optimal effective concentration of the 50% ethanol extract of AI, AM and TC on various physical and biochemical parameters of colonic damage and inflammation induced by AA and TNBS do indicate the effective healing effects (Both physical as well as Biochemical parameters). The above extracts have not any acute toxicity in mice and seemed to be safe.

15. **Procurement/usage of Equipment**: Purchased and reported in first report. All the instruments are in good working condition and used as and when required.

16. **Manuscript for Publication** (300 words for publication in Council's Bulletin): Annexure III

**ANNEXURE I**

1. **Aims & objectives of the scheme-** The present study will be done to evaluate the healing and protective effects of extracts (Ethanolic and aqueous) of *Aegle marmelos (fruit), Terminalia chebula* (fruit) and *Azadirachta indica* (Leaves) on i) experimental ulcerative colitis (UC), and standardization of the extract (finger printing) and safety profile of the extracts following Good laboratory practice and OECD guidelines.

**2. Period of work done:** 1st January 2011 to 31st December 2011

**3. First report:** First year report of the project includes detailed work done on the effects of 50% ethanolic extract of above plants against acetic acid (AA)-induced colitis in rats. It includes effects of the extracts on colonic mucosal damage and weight, adhesions, weight gain, food and water intake, fecal output and study of various biochemical parameters like antioxidants (Superoxide dismutase, catalase and reduced glutathione), free radicals (lipid peroxidation and nitric oxide) and pro-inflammatory marker (myeloperoxidase) in the colonic tissue homogenates.

**4. Materials and Methods**

*Animals* — Inbred Charles-Foster (CF) strain albino rats (150-200 g) of either sex, were obtained from the Central animal house of Institute of Medical Sciences, Banaras Hindu University, Varanasi will be kept in the departmental animal house at $26^0 \pm 2^0$ C, 44- 56% RH and 10:14 hr L:D cycle for 1 week before and during the experiments. Animals will be provided with standard rodent pellet diet (Pashu Aahar, Ramnagar, Varanasi) and water will be given *ad libitum*. 'Principles of laboratory animal care' (NIH publication no. 82-23, revised 1985) guidelines will be 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). 6 animals were taken in each group. The animals will be sacrificed with overdose of ether as and when required.

*Plant material* — 1) Big sized, unripe, Bael (*Aegle marmelos*) 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.

2) Fruit of Haritiki (*Terminalia chebula*) was collected in the months of October to February (Ayurvedic Gardens, Banaras Hindu University/Rajiv Gandhi South Campus, B.H.U.). The fruit pulp was cut into small pieces and dried at room temperature and powdered and stored for further use.

3) Leaves of *A. indica* were collected in the months of March to May (Ayurvedic Gardens, Banaras Hindu University). They were dried in shade 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 extracts*

*Aegle marmelos* (AM)

The aqueous extract of powdered, dried unripe fruit pulp of *Aegle marmelos*, AM (AMW) was prepared by heating 200 ml of distilled water containing 25 g of bael powder on boiling water bath till water content becomes nearly 50 ml. The water extract is filtered and dried to find the solid content i.e. yield of the extract was thus found (16.5%).

The Ethanolic extract 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 Ethanol containing extract so obtained each time were mixed and later dried at $40^0$ C in incubator. The yield of the extracts was 10.1%.

The 50% ethanolic extract of AM (AMWE) was prepared by adding 1 liter of Ethanol in 200 g of dried fine powder of AM. The mixture is shaken at intervals and the extract so obtained is filtered after an interval of two days. The procedure is repeated twice at an interval of two days. The Ethanol containing extract so obtained each time will be

mixed and later dried at $40^0$ C in incubator. The yield of the extracts was 12.7%. Enough quantity of the extract was prepared fresh before use.

*Terminalia chebula* (TC)

The aqueous extract of powdered, dried fruit pulp of *Terminalia chebula*, TC (TCW) was prepared by heating 200 ml of distilled water containing 25 g of Haritiki powder on boiling water bath till water content became nearly 50 ml. The water extract was filtered and dried to find the solid content (yield, 50.3%). The Ethanolic extract of TC (TCE) was prepared by adding 1 liter of Ethanol in 200 g of dried fine powder of TC. 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 Ethanol containing extract so obtained each time were mixed and later dried at $40^0$ C in incubator. The yield of the extracts was 38.9%.

The 50% ethanolic extract of TC (TCWE) was prepared by adding 1 liter of Ethanol in 200 g of dried fine powder of TC. 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 Ethanol containing extract so obtained each time were mixed and later dried at $40^0$ C in incubator. The yield of the extracts was 58.3%. Enough quantity of the extract was prepared fresh before use.

*Azadirachta indica* (AI)

The aqueous extract of powdered, dried leaves of *Azadirachta indica*, AI (AIW) was prepared by heating 200 ml of distilled water containing 25 g of leaves powder on boiling water bath till water content became nearly 50 ml. The water extract was filtered and dried to find the yield content (9.5%).

The Ethanolic extract of AI (AIM) was prepared by adding 1 liter of Ethanol in 200 g of dried fine powder of AI. 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 Ethanol containing extract so obtained each time were mixed and later dried at $40^0$ C in incubator. The yield of the extracts was noted (6.5%).

The 50% ethanolic extract of AI (AIWE) will be prepared by adding 1 liter of Ethanol in 200 g of dried fine powder of AI leaves. 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 Ethanol containing extract so obtained each time were mixed and later dried at $40^0$ C in incubator. The yield of the extracts was noted (10.2%). Enough quantity of the extract was prepared fresh before use.

**Treatment protocol**

Aqueous, 50% ethanolic and ethanolic extracts of *Aegle marmelos* , *Terminalia chebula* and *A. indica* and standard UC protective drug, sulfasalazine were suspended in 0.5% carboxy methyl cellulose (CMC) in distilled water and were given in the volume of 1ml/100 g body weight. The test extracts were given orally once daily for a period of 17 days (3 days before and 14 days after the induction of UC) or 14 days (after the induction of UC) to study the protective and healing effects respectively of the test extracts of the above plants and standard UC protective drug, sulfasalazine.

**Experimental Ulcerative colitis** (UC) was produced by intracolonic administration of acetic acid (AA, 10%, 0.2 ml/rat) (http://herbalmedicine.suite101.com) given per rectally. An initial dose response study was undertaken against AA-induced colitis with an orally administered 50% ethanolic extracts of *Aegle marmelos* (AMWE; 100, 200 and 400 mg/kg), *Terminalia chebula* (TCWE; 300, 600 and 1200 mg/kg), *A. indica* (AIWE; 250, 500 and 1000 mg/kg) and standard UC protective drug, sulfasalazine (SFS, 100 mg/kg, po). They were given orally for a period of 14 after the induction of UC to study the dose response effect of the test extracts of the above plants on colonic mucosal damage score, weight and adhesions. An optimal effective healing dose of AIWE (500 mg/kg), AMWE (200 mg/kg) and TCWE (600 mg/kg) was then selected for further work. Later on a comparative study was done using their above optimal doses of aqueous (W), 50% ethanolic (WE) and pure ethanolic (E) extracts of AI, AM and TC on healing effects on colonic damage score and weight when these extracts were given to rats, orally, once daily for 14 days after induction of colitis with AA. In another set of experiment we also tried to find out the difference if any, between the protective (17 days treatment, 3 days before and

14 days after the induction of colitis) and curative (14 days treatment after the induction of colitis) effects of the 50% ethanolic extracts of the above plants.

After confirming the optimal doses of the extract of these plants and finding no difference between the protective and curative treatments, we then did a comprehensive study with 50% ethanolic extracts of these plants only. Their effects were studied on the number of faecal output with or without blood/mucus, food and water intake and body weight. The biochemical parameters studied were the estimation of i) oxidative free radicals–lipid peroxidation (LPO) and nitric oxide (NO), ii) and antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione and iii) inflammatory marker like myeloperoxidase (MPO) in colonic tissue and pathological changes (both macroscopic and microscopic) by examination of 10 cm distal part of colon of rats after 14 days of treatment in normal saline control (NS control), AA and Test extracts + AA groups. The results of the treatment were compared with that of standard drug, sulfasalazine (SS).

**Acetic acid (AA)-induced colitis study:**

Experimental colitis was produced in rats after administration of single dose of 0.2 ml of 10 % acetic acid intra-rectally [1] into 10 cm of the proximal colon to study the extent of colonic damage score and inflammation which led to various physical and biochemical changes in the colonic tissue that includes physical parameters like diarrhea/increased fecal output, presence of blood/mucous, changes in body weight, food and water intake done on $14^{th}$ day of experiment while other physical (colonic tissue damage, weight and adhesions) [2] and biochemical parameters (antioxidants, free radical status and MPO) were done on $15^{th}$ day of experiment in 18 hour fasted rats. The details of the study with the extracts on following physical and biochemical parameters studied are as follows-

**a) Effects on colonic damage, inflammation and adhesions**:

Untreated rats, receiving 1% CMC orally daily through an orogastric tube, were given normal saline instead of AA in the colon intrarectally (negative control group). They did not show any colonic mucosal damage or adhesions at $15^{th}$ 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 mg/cm (Table 1; Fig. 1). The AA group received 1% CMC orally daily as

above but was given AA in the colon intrarectally in the dose mentioned above. Acetic acid treatment led to significant increase in colonic mucosal damage score (5.78 ± 0.22, P <0.001) and adhesions (61.1%) and increase in colonic weight to 52.7% i.e. from 171.0 mg/cm to261.2 mg/cm compared with untreated rat group values indicating an extensive colonic tissue damage, inflammation together with adhesions due to direct necrotic effect of acetic acid on colonic mucosal tissue. Subsequently graded dose-response effect using AIWE, AMWE and TCWE were observed on above parameters of colonic damage induced by acetic acid to see any healing effects of the above mentioned extracts (Table 1, Fig. 2).

AIWE was given in graded doses of 250, 500 and 1000 mg/kg for 14 days, once daily, orally as suspension in 1% CMC. First dose of AIWE was given 4 hours after induction of colitis with acetic acid on day 1 and then administered once daily till 14 days while, the experiment was conducted on $15^{th}$ day of experiment. AIWE showed dose-dependent decrease in damage score from 25.1% to 74.1% (P <0.01 to P < 0.001), colonic weight from 20.7% to 42.3% (P <0.01 to P < 0.001) and tissue adhesions from 27.3% to 54.5% (Table 1; Figs. 1, 2). Similarly AMWE when given in graded doses of 100, 200 and 400 mg/kg for 14 days after the induction of colitis with AA, once daily, orally as suspension in 1% CMC showed decrease in colonic damage score, colonic weight and adhesions from 10.7% to 73.0% (P < 0.001), 15.8% to 30.8% (P < 0.001) and 54.5% to 81.8% respectively (Table 1; Figs. 1, 2). Again graded doses of TCWE (300, 600 and 1200 mg/kg), when given for 14 days, after the induction of colitis with AA, once daily, orally as suspension in 1% CMC, showed decrease in colonic damage score and weight and adhesions from 43.4% to 68.3% (P < 0.001), 25.4% to 39.1% (P < 0.001) and 54.5% to 81.8% respectively (Table 1; Figs. 1, 2).

From the above dose response study, AIWE (500mg/kg), AMWE (200 mg/kg) and TCWE (600 mg/kg) showing good healing effects on colonic mucosal damage score and inflammation were selected for future studies. Further their effects were comparable with sulfasalazine, a known drug for treatment of ulcerative colitis (positive control) (Table 1; Figs. 1, 2).

A comparative study done with aqueous (W), ethanolic (E) and 50 % ethanolic (WE) extracts of *A. indica* (AI, 500 mg/kg) on colonic mucosal damage score, colonic weight and adhesions induced by acetic acid indicated similar healing effects in terms of decrease in colonic damage score (63.6 to 75.8%), colonic weight (27.5 to 44.2%) and adhesions (19.3 to 38.4%). However, AIWE showed better effects on the above parameters compared to its aqueous (AIW) or ethanolic (AIE) extract and its effect was comparable with sulfasalazine, a known drug for treatment of ulcerative colitis (positive control) (Table 2, Fig. 3). Again comparative study done with aqueous (W), ethanolic (E) and 50 % ethanolic (WE) extracts of *Aegle marmelos* (AM, 200 mg/kg) on colonic mucosal damage score, colonic weight and adhesions induced by acetic acid indicated similar healing effects as found with *A. indica* in terms of decrease in colonic damage score (51.4 to 75.7%), colonic weight (26.1 to 26.5%) and adhesions (19.3 to 57.7%). However, AMWE showed better effects on the above parameters compared to its aqueous (AMW) or ethanolic (AME) extracts and its effect was comparable with sulfasalazine, a known drug for treatment of ulcerative colitis (Table 3, Fig. 4). A comparative study done with aqueous (W), ethanolic (E) and 50 % ethanolic (WE) extracts of *Terminalia chebula* (TC, 600 mg/kg) on colonic mucosal damage score, colonic weight and adhesions induced by acetic acid indicated similar healing effects in terms of decrease in colonic damage score (67.7 to 71%), colonic weight (34.3 to 38.7%) and adhesions(19.3 to 57.7%). However, TCWE showed better effects on the above parameters compared to its aqueous (TCW) or ethanolic (TCE) extracts and its effect was comparable with sulfasalazine, a known drug for treatment of ulcerative colitis (Table 4, Fig. 5).

A comparative evaluation was also done on the selected doses of AIWE (500 mg/kg), AMWE (200 mg/kg) and TCWE (600 mg/kg) for studying their preventive (17 days treatment, 3 days before and 14 days after induction of colitis with AA) effects with the 14 days post AA-induced colitis groups. The result indicated no difference on colonic mucosal damage score, colonic weight and adhesions induced by acetic acid in terms of decrease in colonic damage score, colonic weight and adhesions (Table 5). From the results of the above studies, we finally selected AIWE (500 mg/kg), AMWE (200 mg/kg) and TCWE (600 mg/kg) for future studies on macroscopic and microscopic, physical

(diarrhea, fecal output and body weight) and biochemical (free radicals and antioxidant status) parameters when given for 14 days after the induction of colitis with AA.

**b) Histology study**

i) *Macroscopic study*

The picture in Fig. 6a showed the structures of NS-treated rats treated with NS enema. The picture in Fig. 6b showed the colon of AA-treated rats and significant hydropsia, necrosis, erosion and ulceration were seen. The pictures in Figures 6 c, d, e and f showed the colons with AA-induced colitis treated with AIWE, AMWE, TCWE and SS respectively. The severity of hydropsia, necrosis and ulceration were significantly reduced by all the above treatments. The results of above extracts-treated rats were comparable with that of SS-treated rats.

ii) *Microscopic study*

The photomicrographs of colon shown in Figures 7a-f provided convincing evidence for the protective effects of AIWE, AMWE, TCWE and SS on colitis induced by acetic acid in the rats. The pictures in Fig. 7a showed the morphology of colon of NS enema treated colon of rats treated orally with 1% CMC. The structure was relatively normal and clear with intact epithelia, normal glands and abundant goblet cells except a small amount of inflammatory cells infiltration in the mucosa. Fig. 7b showed the photomicrographs of the AA-treated colon rat treated orally with 1% CMC. The cryptae formations were deformed and the epithelia were not intact. There were distorted glands, loss of goblet cells, and lymphocytic infiltration in mucous layer and edema in submucosa of the colon. Figs. 7c-f showed the photomicrographs of the AA-treated colon rat treated orally with either AIWE or AMWE or TCWE or SS. The treatments with the extracts/SS showed improvement in the structures with near intact epithelia and normal glands. The infiltration of lymphocytes was decreased but still visible.

**c) Effects on diarrhea, fecal output and presence of blood or mucous:**

10% acetic acid 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 (Table 6). They also showed increased fecal output from day 2 onwards (146%). The intensity of fecal output gradually increased over day 4 onwards and at day 14 it was 140.4% compared with Day 0 frequency. Administration of AIWE (500 mg/kg), AMWE (200 mg/kg) and TCWE (600 mg/kg) once daily for 14 days in AA treated rats showed decrease in stool frequency from day 4 onwards which was comparable with sulfasalazine (100 mg/kg) (Table 7, Fig. 8).

**d) 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 14$^{th}$ day of study. Significant decrease on body weight was observed from 6$^{th}$ day onwards as compared with untreated normal animals (Table 8). Treatment with 500, 200 and 600 mg/kg dose of AIWE, AMWE and TCWE respectively for 14 days reversed the decrease trend in body weight suggestive of beneficial effects of the test extracts. The results of the above extracts on body weight showed a similar effect as shown by sulfasalazine-treated rats (Table 8, Figs. 9). However, little or no change was observed on food and water intake between the AA-treated and AIWE, AMWE, TCWE and SS treated animals from 0 day to 14$^{th}$ day of study treatments (Tables 9, 10).

**e) Effects on colonic mucosal free radicals and antioxidant status:**

The effects of AIWE (500 mg/kg), AMWE (200 mg/kg) and TCWE (600 mg/kg) on various biochemical paradigms related to the inflammatory process and healing were estimated in mucosal incubates following induction of colitis by acetic acid. Antioxidant enzymes play an important role in healing and so will be effective in colitis so level of antioxidant parameters like level of superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) were estimated in colonic mucosal homogenates from the normal, AA-treated and extracts treated AA-induced colitis. Further free radicals lipid peroxidation (LPO) and nitric oxide (NO), which are important parameters for tissue damage, were also estimated in the colonic mucosal homogenates from the normal, AA-treated and extracts treated AA-induced colitis.

i) Effects on free radicals- LPO and NO

The level LPO [3] and NO [4] were estimated in the colonic mucosal incubates both in normal untreated and AA-treated rats. The animals were sacrificed with overdose of ether; the part of colon up to 10 cm from rectum is taken out. The colonic scrapings thus obtained from the affected areas on day 15$^{th}$ of experiment were homogenized for estimation of both LPO and NO following the standard procedures.

AA did not cause any change in protein content of the colonic mucosa expressed as mg/g wet tissue while it enhanced both LPO and NO expressed either as mmol/g wet tissue or mmol/mg protein compared to normal untreated rats (Table 11, Fig. 10). AIWE (500 mg/kg), AMWE (200 mg/kg), TCWE (600 mg/kg) and SS (100 mg/kg) showed reversal of levels of both LPO and NO near to the untreated normal rats (Table 11, Fig. 10). The effect on free radicals by AIWE, AMWE and TCWE were comparable with SS.

ii) Effect on antioxidants- SOD, CAT and GSH

AA treated animals showed significant decrease in both SOD [5], CAT [6] and GSH [7] levels in the colonic mucosal incubates when expressed either as U (SOD & CAT) or nmol (GSH) per g wet tissue weight or per mg protein compared to normal untreated rats (Table 11, Fig. 10). AIWE (500 mg/kg), AMWE (200 mg/kg), TCWE (600 mg/kg) and SS (100 mg/kg) were given for 14 days after AA-induction of colitis reversed the above changes in SOD, CAT and GSH levels near to normal untreated group (Tables 11, 12; Figs. 10-12).

iii) Effect on Myeloperoxidase enzyme, MPO (Inflammatory marker)

AA treated animals showed significant increase in MPO [8] level in the colonic mucosal incubates when expressed either as U/g wet tissue weight or mU/ mg protein compared to normal untreated rats (Table 12, Fig. 11). AIWE (500 mg/kg), AMWE (200 mg/kg), TCWE (600 mg/kg) and SS (100 mg/kg) were given for 14 days after AA-induction of colitis reversed the above changes in MPO level near to normal untreated group (Table 12, Fig. 13).

The results of present study with the extracts of AI, AM and TC on various physical and biochemical parameters of colonic damage and inflammation induced by AA do indicate the effective healing effects of 50% ethanolic extracts of all plants. Further

50% ethanolic extracts of the plants seemed to be more effective compared to either ethanolic or aqueous extracts indicating the validity of use of 50% ethanolic extracts as practice in Ayurveda.

**References**


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**ANNEXURE II**

1. **Aims & objectives of the scheme-** The present study will be done to evaluate the healing effects of extracts of *Aegle marmelos (fruit), Terminalia chebula* (fruit) and *Azadirachta indica* (Leaves) on i) TNBS induced experimental ulcerative colitis (UC), and standardization of the extract (finger printing) and their safety profile following Good laboratory practice and OECD guidelines.

**2. Period of work done:** 1st January 2012 to 30th June 2013

**3. Second report:** Second year report of the project includes detailed work done on the effects of 50% ethanolic extract of above plants against Trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats. It includes effects of the extracts on colonic mucosal damage and weight, adhesions, weight gain, food and water intake, diarrhoea (fecal output) and study on various biochemical parameters like antioxidants (superoxide dismutase, catalase and reduced glutathione), free radicals (lipid peroxidation and nitric oxide) and acute inflammatory marker (myeloperoxidase) in the colonic tissue homogenates. Phytochemical and acute toxicity studies have been done with the extracts and included in the report.

**4. Materials and Methods**

*Animals* — Inbred Charles-Foster (CF) strain albino rats (180-200 g) of either sex, were obtained from the Central animal house of Institute of Medical Sciences, Banaras Hindu University, Varanasi will be kept in the departmental animal house at $26^{o} \pm 2^{o}$ C, 44- 56% RH and 10:14 hr L:D cycle for one week before and during the experiments. Animals will be provided with standard rodent pellet diet (Pashu Aahar, Ramnagar, Varanasi) and water will be given *ad libitum*. 'Principles of laboratory animal care' (NIH publication no. 82-23, revised 1985) guidelines will be 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). 6 animals were taken in each group. The animals will be sacrificed with overdose of ether as and when required.

*Plant material* — 1) Big sized, unripe, Bael (*Aegle marmelos*) 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.

2) Fruit of Haritiki (*Terminalia chebula*) was collected in the months of October to February (Ayurvedic Gardens, Banaras Hindu University/Rajiv Gandhi South Campus, B.H.U.). The fruit pulp was cut into small pieces and dried at room temperature and powdered and stored for further use.

3) Leaves of *A. indica* were collected in the months of March to May (Ayurvedic Gardens, Banaras Hindu University). They were dried in shade 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 extracts*

*Aegle marmelos* (AM)

The 50% ethanolic extract of AM (AME) was prepared by adding 1 liter of 50% ethanol in 200 g of dried fine powder of AM. The mixture is shaken at intervals and the extract so obtained is filtered after an interval of two days. The procedure is repeated twice at an interval of two days. The Ethanol containing extract so obtained each time will be mixed and later dried at $40^{o}$ C in incubator. The yield of the extracts was 12.7%. Enough quantity of the extract was prepared fresh before use.

*Terminalia chebula* (TC)

The 50% ethanolic extract of TC (TCE) was prepared by adding 1 liter of 50% ethanol in 200 g of dried fine powder of TC. 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 Ethanol containing extract so obtained each time were mixed and later dried at $40^{o}$ C in incubator. The yield of the extracts was 58.3%. Enough quantity of the extract was prepared fresh before use.

*Azadirachta indica* (AI)

The 50% ethanolic extract of AI (AIE) will be prepared by adding 1 liter of 50% ethanol in 200 g of dried fine powder of AI leaves. 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 Ethanol containing extract so obtained each time were mixed and later dried at $40^{o}$ C in incubator. The yield of the extracts was noted (10.2%). Enough quantity of the extract was prepared fresh before use.

**Treatment protocol**

AME, TCE, AIE 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. The test extracts were given orally once daily for a period of 14 days to study the healing effect of the test extracts of the above plants and standard UC protective drug, sulfasalazine.

**Experimental colitis**

Doses of AME (200 mg/kg), TCE (600 mg/kg), AIE (500 mg/kg) and standard UC protective drug, SS (100 mg/kg) were selected as per our previous reported project/published work. Colitis was produced by administration of TNBS[1] given per rectally into 10 cm of the proximal colon. Rats were either given intracolonic normal saline (NS, 0.4 ml/rat, negative control) or TNBS alone (40 mg/0.4 ml of 40% ethanol/rat, control) or TNBS plus oral AME (200 mg/kg)/TCE (600mg/kg)/AIE (500mg/kg)/SS (100mg/kg, SS, positive control drug) to study the extent of colonic damage score and inflammation which led to various physical and biochemical changes in the colonic tissue that includes physical parameters like diarrhea (increased fecal output), changes in body weight, food and water intake seen on day 0, $7^{th}$ and $14^{th}$ day of experiment while other physical (colonic tissue damage, weight and adhesions)[1] and biochemical parameters (antioxidants, free radical and MPO enzyme level)[2-8] were estimated on $15^{th}$ day of experiment in 18 hour fasted rats. The details of the study with the extracts on following physical and biochemical parameters studied are as follows-

**Effect on diarrhoea**

Diarrhoea was observed in intracolonic TNBS-induced colitis and after administration of oral CMC/test extracts/SS. The effects were seen on day 0 (start of the experiment) and $7^{th}$ and $14^{th}$ day of the experiment. The result of Test extracts/SS treated groups were compared with TNBS group.

**Effect on body weight, food intake and water intake**

The above parameters were measured on day 0 (start of the experiment) and $7^{th}$and $14^{th}$ day of the experiment. Each rat was individually weighed using standard rat weighing machine and their respective weights were noted down. Similarly a measured weight of enough food and water was given to each rat housed individually in the iron cages (8 x 11 x 7 cubic inches) at a fixed time of day and next day the amount of food and water left was calculated for individual rat. The result of Test extracts/SS treated groups were compared with TNBS group.

**Effect on colonic damage and inflammation**

All scorings of damage and excision of tissue samples were performed by an observer unaware of the treatment group. The rats in the various treatment groups were randomized before being sacrificed. The rats were weighed and sacrificed by an over dose of ether and proximal 8 cm of colon was removed. The colon was opened by a longitudinal incision, rinsed with tap water and pinned out on a wax block. Macroscopically visible damage was scored on a 0-10 scale using the scoring system as described by Morris and

associates, 1989[1], which takes into consideration the severity and number of ulcers in terms of tissue damage score, thickening and adhesions (signs of inflammation). Subsequently 8 cm of colon were taken for measurement of weight and weight was then expressed as mg/cm length of individual rat. The result of intracolonic TNBS+ oral CMC group was compared with NS intracolonic + oral CMC group while, the result of TNBS + Test extracts/SS treated groups were compared with TNBS group.

**Histopathology of Colon**

Histopathology of the colon was done in all the groups on 15$^{th}$day of experiment to know the status of healing. Approximately 0.5cm x 0.5cm of colon was taken and fixed in 10% buffered formalin and paraffin embedded. 4-6 μm thick sections were stained with Hemotoxylin and Eosin stain for histological evaluation and examined under microscope at x100 magnification.

**Biochemical analysis**

On 15$^{th}$ day of experiment the animals were sacrificed and colon of each rat was taken out and washed with cold normal saline. Antioxidants enzymes superoxide dismutase, SOD[2], catalase, CAT[3] and non-enzyme, reduced glutathione, GSH[4]; free radicals *i.e.* lipid peroxidase, LPO[5] and nitric oxide, NO[6]; acute inflammatory marker, myeloperoxidase, MPO[7] and protein[8] were estimated in colonic mucosal homogenates following the standard procedures. LPO levels were estimated in terms of malondialdehyde (MDA) released during lipid peroxidation. Nitrites and nitrates are formed as end products of reactive nitrogen products during NO formation which are measured by using Griess reagent. SOD activity was estimated by its ability to inhibit reduction of nitro blue tetrazolium to blue coloured formazan in presence of phenazine metha sulphate (PMS) and NADH. One unit (U) of enzyme activity is defined as enzyme concentration required to inhibit the chromogen conversion by 50%.CAT measurement was done based on the ability of catalase to oxidize hydrogen peroxide.One unit (U) of catalase is the enzyme, which decomposes one mM of hydrogen peroxide per min at 25$^{o}$C. 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. MPO activity was determined as an indicator of polymorphous nuclear leucocyte accumulation. MPO activity was estimated by its ability to inhibit reduction of nitro blue tetrazolium to blue coloured formazan in presence of phenazine metha sulphate (PMS) and NADH. One unit (U) of enzyme activity is defined as enzyme concentration required inhibiting the chromogen conversion by 50%.

**Antimicrobial activity**

*In vitro* antibacterial susceptibility test of TCE/AME/AIE 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[9] 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[10].

**Preliminary phytochemical screening and High Performance Liquid Chromatography (HPLC) study**

AIE, AME and TCE was subjected to qualitative tests for the identification of tannins, glycosides, carbohydrate, fixed oil and fats, protein and amino acids, phenolic compounds, gum and mucilage, saponins, alkaloids, flavonoids and triterpenoids as per the standard procedure[11]. Extract samples were used for qualitative and quantitative analysis of flavonoids by HPLC-PDA with a Shimadzu (Japan) LC-10 system comprising an LC-10AT dual pump system, an SPD-M20A PDA detector, and rheodyne injection valve furnished with a 20µL sample loop. Compounds were separated on a 4.6 x 250 mm, i.d., 5µM pore size Merck RP-C18 column protected with guard column of same chemistry. The mobile phase was a gradient prepared from 0.5% (v/v) phosphoric acid in HPLC-grade water (component A) and methanol (component B). Before use the components were filtered through 0.45-µM nylon filters and de-aerated in an ultrasonic bath. Analysis of the sample was carried out as described by Niranjan *et al.* (2011)[12] with modifications[13]. Elution was carried out at a flow rate of 0.8 mL/min with 0.5% phosphoric acid as solvent A and methanol as solvent B using a gradient elution in 0-5 min. with 75-70% A, 5-10 min. with 70-50% of A, 10-15 min. with 50-20% of A, 15-25 min. with 20-80% of A.

**Limit test (acute toxicity) study in mice**

Six adult Swiss albino mice of either sex (3 males and 3 females), weighing between 25 to 30 g fasted overnight, were used for acute toxicity study as per OECD guideline. Suspension of all the extracts were orally administered maximum of 2 g/kg stat dose (3.33 times of the optimal effective dose of TCE, 600 mg/kg; 10 times the optimal dose of AME, 200 mg/kg and 4 times the optimal dose of AIE, 500 mg/kg) to mice. Subsequent to extracts administration, animals were observed closely for first four hours, for any toxicity manifestation, like increased motor activity, salivation, convulsion, coma and death. Subsequently observations were made at regular intervals for 24 h. The animals were under further investigation up to a period of two weeks[14].

**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 ± SEM. $P < 0.05$ was considered significant.

## Results

### Effects on diarrhea

TNBS rats showed an increase in fecal output (control output, 2.33 ± 0.13 g/100 g body weight) from the beginning of experiment (day 0) to 49.4% and 57.5% ($P < 0.01$) at day 7 and 14 respectively. TNBS+AME rats showed an increase in fecal output by 27.7% ($P < 0.05$) and 16.9% from its day 0 value indicating a decrease in fecal output by 21.6% and 40.6% ($P < 0.05$) compared with TNBS group at day 7 and 14 respectively. TNBS + AIE-treated rats showed an increase in fecal output by 30.4% ($P < 0.05$) and 23.3% ($P < 0.05$) from its day 0 value indicating a decrease in fecal output by 19.0 and 34.2% ($P < 0.05$) compared with TNBS group at day 7 and 14 respectively. TCE treated rats showed an increase in fecal output by 24.3% and 25.2% from its day 0 value indicating a decrease in fecal output by 25.1% and 32.3% compared with TNBS group at day 7 and 14 respectively while, SS treated rats showed an increase in fecal output by 37.4% ($P < 0.05$) and 15.7% from its day 0 value indicating a decrease by 12.0% and 41.8% ($P<0.01$) compared with TNBS group at day 7 and 14 respectively (Table 1, Fig. 1).

### Effects on body weight changes and food and water intakes:

TNBS enema rats showed a decrease in body weight from 199.4 ± 2.19 g at day 0 to 182.8 ± 3.77 (8.3% decrease) ($P < 0.01$) and 171.1 ± 2.71 (14.2% decrease) ($P < 0.001$) at $7^{th}$ and $14^{th}$ day respectively compared to day 0 value. AME-, AIE- and TCE-treated TNBS-induced colitis rats showed an increase in body weight by 8.9% and 12.5% ($P < 0.01$), 7.1% and 10.9% ($P < 0.05$) and 11.8% and 15.2% ($P<0.01$) at $7^{th}$ and $14^{th}$ day respectively from their respective day 0 weight. TNBS+SS treated rats showed an increase in body weight by 10.6% and 16.4% ($P < 0.001$) at $7^{th}$ and $14^{th}$ day respectively from day 0 weights. However, all the extracts- and SS-treated rats showed significant increase in the body weight compared to respective $7^{th}$ and $14^{th}$ TNBS treated group alone (Table 2, Fig. 2).

Mild or no change was found in the food and water intake at $7^{th}$ or $14^{th}$ day of study amongst the groups (Table 3, 4).

### Effects on colonic damage and inflammation

Untreated rats, receiving 0.5% CMC orally daily through an orogastric tube, were given normal saline (NS) instead of TNBS in the colon intrarectally (negative control group). They did not show any colonic mucosal damage or adhesions at $15^{th}$ day of experiment while, the colonic weight (8 cm of proximal colon) expressed as mg/cm of colon was found to be 158.3 ± 6.37 mg/cm. The TNBS group received 0.5% CMC orally daily as above but was given TNBS in the colon intrarectally in the dose mentioned above. TNBS given intrarectally led to significant increase in colonic mucosal damage score (5.17

± 0.31, $P < 0.001$), adhesions (5/6 rats, 83.3%) and weight to 248.8 ± 6.7 mg/cm (57.2% increase, $P < 0.001$) compared with NS group (Table 5; Fig. 3).

Rats treated with oral AME (200 mg/kg) showed decrease in colonic damage score, colonic weight and adhesions by 67.7% ($P < 0.001$), 29.1% ($P < 0.001$) and 40.0% respectively. AIE (500 mg/kg)-treated rats showed decrease in damage score (64.6%, $P < 0.001$), colonic weight (34.6%, $P < 0.001$) and tissue adhesions (40.0%) in rats. Again, TCE (600 mg/kg)-treated rats showed decrease in colonic damage score and weight and adhesions by 61.3% ($P < 0.001$), 27.0% ($P < 0.001$) and 60.0% respectively. SS-treated rats showed a decrease in colonic damage score, colonic weight and adhesions by 77.4% ($P < 0.001$), 33.5% ($P < 0.001$) and 80.0% (1/6 rat) respectively compared with TNBS group. Further, their effects were comparable with sulfasalazine, a known drug for treatment of ulcerative colitis (positive control) (Table 5; Fig. 3).

**Histopathological study**

i) *Macroscopic study*

The picture in Fig. 4a showed the macroscopic changes seen in normal saline (NS) enema treated colon with oral 0.5% CMC, indicating normal morphology. The picture in Fig. 4b showed macroscopic changes in the rat colon of 2, 4, 6-trinitrobenzene sulfonic acid-induced colitis, treated with oral CMC showing necrosis, erosion, hydropsia and ulceration. The pictures in Figures 4 c, d, e and f showed the colons with TNBS-induced colitis treated with AIE, AME, TCE and SS respectively. The severity of hydropsia, necrosis and ulceration were significantly reduced by all the above treatments. The results of above extracts-treated rats were comparable with that of SS-treated rats.

ii) *Microscopic study*

The photomicrographs of colon shown in Figures 5a-f provided convincing evidence for the healing effects of AIE, AME, TCE and SS on colitis induced by TNBS in the rats. The pictures in Fig. 5a showed the morphology of colon of NS enema colon of rats treated orally with 0.5% CMC. The structure was relatively normal and clear structure with intact mucosa and sub mucosa. Fig. 5b showed the photomicrographs of the TNBS enema colon rat treated orally with 0.5% CMC showing crypt destruction with severe cryptitis (blue arrow), ulceration with eroded mucosa (brown arrow), lymphoplasmacytic infiltrate (white arrow) and transmural inflammation (Yellow arrow). Figs. 5c-e showed the photomicrographs of the TNBS enema rat colon treated orally with AIE/AME/TCE showing regenerative mucosa with mild crypt distortion and mild lymphoplasmacytic infiltrate in the lamina propria with edematous submucosa. Fig. 5f showed the photomicrographs of the TNBS enema rat colon treated orally with SS showing intact mucosa with minimal lymphoplasmacytic infiltrate in the lamina propria. The treatments

with the extracts/SS showed improvement in the structures with near intact epithelia and normal glands. The infiltration of lymphocytes was decreased but still visible.

**Effects on colonic mucosal free radicals and antioxidant status:**

The effects of AIE (500 mg/kg), AME (200 mg/kg) and TCE (600 mg/kg) on various biochemical paradigms related to the inflammatory process and healing were estimated in mucosal incubates following induction of colitis by TNBS. Antioxidant enzymes play an important role in healing and so will be effective in colitis so level of antioxidant parameters like level of superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) were estimated in colonic mucosal homogenates from the normal (NS enema), TNBS enema and extracts treated TNBS-induced colitis. Further free radicals lipid peroxidation (LPO) and nitric oxide (NO), which are important parameters for tissue damage, were also estimated in the colonic mucosal homogenates from the normal, TNBS enema and extracts treated TNBS-induced colitis. The animals were sacrificed with overdose of ether; the part of colon up to 10 cm from rectum is taken out. The colonic scrapings thus obtained from the affected areas on day $15^{th}$ of experiment were homogenized for estimation of above parameters following the standard procedures.

i) Effects on free radicals- LPO and NO

The levels LPO and NO were estimated in the colonic mucosal incubates both in normal colon (NS enema) and TNBS enema rat colon. TNBS did not cause any change in protein content of the colonic mucosa expressed as mg/g wet tissue while it enhanced both LPO and NO expressed either as mmol/g wet tissue or mmol/mg protein compared to normal untreated (NS enema) rats. AIE (500 mg/kg), AME (200 mg/kg), TCE (600 mg/kg) and SS (100 mg/kg) showed reversal of levels of both LPO and NO near to the oral CMC-treated, intracolonic NS rats. The effect on free radicals by AIE, AME and TCE were comparable with SS (Table 6, Fig. 6).

ii) Effect on antioxidants- SOD, CAT and GSH

TNBS treated animals showed significant decrease in both SOD, CAT and GSH levels in the colonic mucosal incubates when expressed either as mU (SOD and CAT) or nmol (GSH) per g wet tissue weight or per mg protein compared to normal untreated rats. AIE (500 mg/kg), AME (200 mg/kg), TCE (600 mg/kg) and SS (100 mg/kg) when given for 14 days after TNBS-induction of colitis reversed the above changes in SOD, CAT and GSH levels near to the oral CMC-treated, intracolonic NS rats (Table 6; Figs. 7, 8).

iii) Effect on Myeloperoxidase enzyme, MPO (Inflammatory marker)

TNBS treated animals showed significant increase in MPO[8] level in the colonic mucosal incubates when expressed as mU/ mg protein compared to normal untreated rats. AIE, AME, TCE and SS when given for 14 days after TNBS-induction of colitis reversed the above changes in MPO level near to oral CMC-treated, intracolonic NS rats (Table 6, Fig. 9).

**Antimicrobial activity**

*In vitro* antibacterial susceptibility test of against various intestinal pathogens i.e. *Escherichia coli* ATCC 25922, *Shigella sonnei*, *Shigella boydii* and *Shigella flexneri* with serial concentrations of 50, 100, 150 and 200 mg/ml of AME/AIE/ TCE showed zone of inhibition >10 mm with their dose of 200 mg/ml. MIC values with AME and AIE were 12.5, 6.25, 6.25 and 1.57 mg/ml; and 12.5, 6.25, 6.25 and 3.12 mg/ml respectively against *Escherichia coli*, *Shigella sonnei, Shigella boydii* and *Shigella flexneri* while, TCE showed MIC values against the above organisms as 6.25, 1.57, 1.57 and 0.79 mg/ml respectively indicating more susceptibility of *Shigella sonnei, Shigella boydii* and *Shigella flexneri* to lower concentrations of TCE (Table 7).

**Preliminary phytochemical screening**

AIE, AME and TCE showed the presence of tannins, glycosides, carbohydrate, fixed oil and fats, protein and amino acids, phenolic compounds, gum and mucilage, saponins, alkaloids, flavonoids and triterpenoids (Table 8).

**High Performance Liquid Chromatography (HPLC)**

AME, AIE and TCE samples were tested for qualitative and quantitative analysis of flavonoids against the known flavonoids namely quercetin, rutin and kaempferol by HPLC-PDA with a Shimadzu (Japan) LC-10 system. The result indicated the presence of flavonoids, rutin in AIE and TCE and quercetin in AME (Fig. 10).

**Acute toxicity study in mice**

AME, AIE and TCE at dose of 2.0 g/kg in adult Swiss albino mice did not show any acute toxicity manifestations like increased motor activity, salivation, convulsions, coma and death in mice, observed up to a period of two weeks.

**Conclusion**

The results of present study with the extracts of AI, AM and TC on various physical and biochemical parameters of colonic damage and inflammation induced by TNBS do indicate the effective healing effects of 50% ethanolic extracts of all plants which could be due to presence of flavonoids as observed in our HPLC study. Further 50% ethanolic

extracts of the plants seemed to be safe and had antibacterial activity better suited in colitis where intestinal pathogens do have a role to play. Thus, the effectiveness of these plant extract both against acetic acid as well as TNBS induced colitis demonstrate the validity of their uses in colitis as practiced in Ayurveda.

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