ORIGINAL ARTICLE

Gastro protective effect of *Agave americana* Linn. leaf extract in indomethacin-induced enterocolitis in rats

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Abstract

Objective: The present study was undertaken to evaluate the gastro protective effect of *Agave americana* Linn. leaf extract in indomethacin-induced enterocolitis in rats using prednisolone as standard drug. **Methods:** Male Wistar rats were pretreated with *A. americana* leaf extract in the dose of 200 and 400 mg/kg orally daily for 7 days. On 8th and 9th day indomethacin was administered (7.5 mg/kg, s.c.) to induce enterocolitis. The treatment was continued until 11th day. On 11th day animals were sacrificed and dissected open to remove ileum, cecum, and colon. Flushed gently with saline, cut open and inflammation was assessed based on the physical parameters, scoring of ulcer, myeloperoxidase (MPO), and lipidperoxidase (LPO) activity. **Results:** *A. americana* leaf extract (200 and 400 mg/kg) showed a significant decrease ($P < 0.001$) in ulcer scores for inflammation. Both prednisolone and *A. americana* have retained total body weight significantly ($P < 0.01$). Significant decrease ($P < 0.001$) in MPO and LPO activity was also observed. Histopathology of extract treated group showed reduced intensity of edema, inflammatory cellular infiltration, crypt damage and ulceration at mucosal and submucosal layers compared to induce control group. **Conclusion:** The above findings prove that, *A. americana* Linn. leaf extract has protective effect against indomethacin-induced enterocolitis.

Key words: *Agave americana*, indomethacin, lipidperoxides, myeloperoxidase, ulcer index

INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) together constitute inflammatory bowel disease (IBD). CD (enterocolitis) may be localized in any part of the digestive tract and affects the entire intestinal wall, whereas UC is confined to the colon and rectum and the inflammation is restrained to the intestinal lining.[1] IBD is a growing worldwide health burden; specifically many developing countries have seen a dramatic rise in the incidence of IBD since 1990.[2] Although etiology of IBD is unknown, it appears that an abnormal response of the mucosal innate immune system to luminal bacteria may trigger inflammation which is perpetual by dysregulation of cellular immunity.[3-5] The most widely accepted hypothesis in the pathogenesis of IBD is that the mucosal immune system shows an aberrant response toward luminal antigens such as dietary factors and/or bacteria in genetically susceptible individuals. The chronic inflammatory process leads to disruption of the epithelial barrier, and the formation of epithelial ulceration. This permits easy access for the luminal microbiota and dietary antigens to cells resident in the lamina propria and stimulates further pathological immune cell responses. Cytokines are essential mediators of the interactions between activated immune cells and non-immune cells, including epithelial, and mesenchymal cells. The clinical efficacy of targeting tumor necrosis factor-alpha (TNF-α) clearly indicates that cytokines are the therapeutic targets in IBD patients.[6] CD and UC are caused by excessive immune reactivity in the gut wall. Analysis

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of the type of immune responses ongoing in diseased gut has revealed important features.[7] CD is disease of young people with a peak incidence between the ages of 10 and 40 years. However, it affects people of any age and 15% of people are over the age of 60 at diagnosis. Up to 240,000 people are affected by IBD in the UK. Symptoms of CD includes diarrhea, right lower quadrant pain, fever, weight loss trans-mural inflammation-large ulcers, rectum spared, skip lesions, fistulae, and strictures.[8]

Immuno-modulators, such as azathioprine, 6-mercaptopurine, methotrexate or cyclosporin, are demonstrating increasing importance against steroid-resistant and steroid-dependent patients.[9] The drugs which are found to be effective are sulfasalazine (azulfidine, salazopyrin) and its 5-aminosalicylic acid derivatives, glucocorticosteroids, immunomodulators/immunosuppressants, and other new potential drugs.[10]

There is widespread belief that the natural products are less toxic when compared to pure chemicals. Phyto-botanical and ethnobotanical research have focused for decades on the search for the single active principle in plants based on the assumption that plant has one or a few ingredients, which determine its therapeutic effects. However, the traditional system of medicine like Ayurveda, traditional Chinese medicine or the European Pharmacotherapy generally assumes that synergy of all ingredients of the plants will bring about the maximum therapeutic efficacy.[11] Recent data suggests that 80% drug molecules are natural products or natural compound inspired.[12]

The leaves of *Agave americana* Linn. (Family - *Agavaceae*) are used as hepatoprotective, antioxidant, antiseptic, and to relieve various liver diseases.[13] *Agave* leaves sap was used internally in case of wounds and inflammation, and it also has anti-inflammatory activity.[14,15]

To best of our knowledge, no scientific data regarding the activity of *A. americana* on IBD is available in literature. Therefore, we have undertaken this work anti-IBD activity in indomethacin-induced enterocolitis in rats.

METHODOLOGY

Drugs and Reagents

Prednisolone, indomethacin, and trichloroacetic acid were purchased from Himedia, Mumbai. Other chemicals were purchased from Ranbaxy.

Plant Material

The fresh leaves of *A. americana* Linn. were collected from surrounding areas of Dharwad, Karnataka and authenticated by Dr. Hebbar, Dharwad. The plant material was washed with running water and ground well using mortar and pestle. The obtained herbal juice was filtered and filtrate was subjected to rotary flash evaporator under reduced pressure to dryness. The dried extract was stored in desiccators until use.[16]

Preliminary Phytochemical Investigation

The *A. americana* leaf extract was subjected to preliminary phytochemical investigation as described in practical pharmacognosy by Kokate and Khandelwal.[17,18]

Animals

Male Wistar rats weighing 150-200 g were used for the present study. For acute toxicity study, female rats were used. The animals were purchased from Sri Venkateshwara Enterprises and maintained in the Animal House of SET's College of Pharmacy, Dharwad for experimental purpose. The animals were maintained under controlled conditions of temperature (22 ± 2°C), humidity (50% ± 5%) and 12-h light-dark cycles. They were fed commercial stock diet and water, *ad libitum*. The animals were housed individually in sanitized polypropylene cages containing sterile paddy husk as bedding. Animals were habituated to laboratory conditions for 48 h prior to the experimental protocol to minimize if any of non-specific stress. All the studies conducted were approved by the Institutional Animal Ethical Committee of SET's College of Pharmacy, Dharwad, Karnataka (Reg. No. 112/1999/CPCSEA) according to prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India.

Determination of Acute Toxicity

The acute oral toxicity study was carried out as per the guidelines set by OECD 423. Animals ($n = 3$) were overnight fasted prior to dosing. The dose level to be used as the starting dose was selected from one of four fixed levels, 5, 50, 300, and 2000 mg/kg b.w. The test substance was administered in a single dose by gavage using intubation canula. Animals were observed individually after dosing at least once during the first 30 min, periodically during the first 24 h, with special attention given during the first 4 h, and daily thereafter, for a total of 14 days. Animals were observed for following profiles.

1. Behavioral profile - Alertness, restlessness, irritability and fearfulness
2. Neurological profile - Spontaneous activities, reactivity, touch and pain response
3. Autonomic profile - Defecation and urination.

After a period of 24 and 72 h animals were observed for death.[19]

Experimental Methods

Indomethacin-induced enterocolitis in rats

The animals were divided into five groups each containing six rats shown in Table 1.

Animals were pre-treated with *A. americana* extract for 7 days. On 8th and 9th day indomethacin (7.5 mg/kg) was administered to induce enterocolitis. Extract administration was continued until 11th day. On the 11th day the animals were sacrificed. Ileum, cecum, and colon were taken out to assess for inflammation based on physical parameters, macroscopic, and microscopic features. Quantification of inflammation was done using biochemical assay (myeloperoxidase [MPO] and lipidperoxidase [LPO]).[20,21]

Evaluation of the Disease

Change in body weight

As the weight loss is one of the clinical symptoms of enterocolitis all the rats from respective groups are weighed each day and the percentage of original weight is used for evaluation.[22]

Evaluation Based on Macroscopic Characters

Scoring of ulcer for ileum, cecum, and colon

The distal 10 cm portion of colon ileum and whole cecum was removed cut longitudinally and cleaned in physiological saline and were scored for macroscopic features using standard scoring pattern. Score for an individual rat was calculated as the combined score of ileum colon and caecum.[21]

Evaluation Based on Microscopic (histological) Characters

Caecum and ileum from each animal were removed and preserved in 10% formalin. The samples were submitted to Jeevan Regional Diagnostic Health Care and Research Centre Pvt. Ltd. (Belgaum, India) for histological examination.

Table 1: Grouping and treatment regimen for animals

Groups	Treatment
1	Normal control (treated with vehicle)
2	Positive control - Indomethacin (7.5 mg/kg, s.c)
3	*A. americana* extract (200 mg/kg)+Indomethacin
4	*A. americana* extract (400 mg/kg)+Indomethacin
5	Prednisolone (2 mg/kg)+Indomethacin

A. americana: Agave americana

Evaluation Based on Biochemical Parameters

Myeloperoxidase (MPO) assay for quantification of inflammation

The cecum was taken weighed and rinsed with ice-cold saline blotted dry weighed and excised. Minced tissue was homogenized in 10 volumes of ice-cold potassium phosphate buffer (pH 7.4) using Remi tissue homogenizer (RQ-127A). The homogenate was centrifuged at 3500 rpm for 30 min at 4°C (Remi centrifuge C23). The supernatant was discarded. 10 ml of icecold 50 mM potassium phosphates buffer (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide and 10 mM ethylenediaminetetraacetic acid was then added to the pellet. It was then subjected to one cycle of freezing and thawing and brief period (15 s) of sonication. After sonication solution was centrifuged at 15,000 rpm for 20 min (Remi centrifuge, R24). MPO activity was measured spectrophotometrically as follows: 0.1 ml of supernatant was combined with 2.9 ml of 50 mM phosphate buffer containing 0.167 mg/ml O-dianisidine hydrochloride and 0.0005% H_2O_2. The change in absorbance was measured spectrophotometrically (Shimadzu UV 1 60A UV-VIS spectrophotometer) at 460 nm. One unit of MPO activity is defined as the change in absorbance per minute by 1.0 at room temperature in the final reaction.[20]

Calculation of MPO activity:

$$\text{MPO activity } (\text{U/g}) = \frac{\text{X}}{\text{Weight of the piece of tissue taken}}$$

$$\text{Where X} = \frac{10 \times \text{changes in absorbance per minute}}{\text{Volume of supernatant taken in the final reaction}}$$

Measurement of colonic LPO concentration

LPO an indicator of mucosal injury induced by reactive oxygen species was measured as thiobarbituric acid reactive substance (TBARS). LPO was measured by the TBA. Briefly 0.5 ml of tissue homogenate prepared were reacted with 2 ml of TBA reagent containing 0.375% TBA 15% trichloroacetic acid and 0.25 N HCl. Samples were boiled for 15 min cooled and centrifuged. Absorbance of the supernatants was spectrophotometrically measured at 532 nm. TBARS concentrations were calculated by the use of 1,3,3,3-tetra ethoxypropane as a standard. The results were expressed as µmol/g wet tissue weight.[21]

Statistical Analysis

All data were expressed as mean ± standard error of the mean of six rats per experimental group. Statistical analysis was performed using Graph pad prism 5.0 statistical software. Parametric one-way analysis of variance followed by Tukey's post-test. The minimal level of significance was identified at $P < 0.05$.

RESULTS

Phytochemical Investigation

The phytochemical investigation of *A. americana* Linn. leaf extract showed the presence of steroids, triterpenoids, saponin glycosides, flavonoids, alkaloids and carbohydrates.

Acute Toxicity Studies

Acute toxicity study revealed that animals showed good tolerance (up to a dosage of 2000 mg/kg b.w) to single doses of *A. americana*. There were no signs and symptoms of restlessness, irritability, fearfulness, pain response, convulsions, defecation, urination, or coma. Therefore, two non-lethal doses (200 and 400 mg/kg b.w) of extract were selected.

Indomethacin-Induced Enterocolitis in Rats

Effect of A. americana Linn. leaf extract on body weight

Weight loss is one of the clinical symptoms of enterocolitis all the rats from respective groups were weighed each day and the percentage of original weight is used for evaluation. Evaluation based on the body weight showed significant ($P < 0.001$) decrease in the body weight of the positive control group compared with normal group [Table 1]. The rats treated with the extract 200, 400 mg/kg and standard group showed significant ($P < 0.01$, $P < 0.001$ and $P < 0.001$, respectively) increase in body weight compared with positive control group. These values suggest that the body weight has been maintained with these animals compared to positive control group [Figure 1].

Ulcer scoring for ileum, cecum and colon

The standard drug and the extract have significantly reduced ($P < 0.001$) ulcer scores compared to positive control [Table 2]. The values of the drug treated group (especially 400 mg/kg) were comparable with standard group. Score for an individual rat was calculated as the combined score of ileum, colon and caecum. Figures 2a-d shows the ulcer score of normal control, induced extract treated, and standard groups.

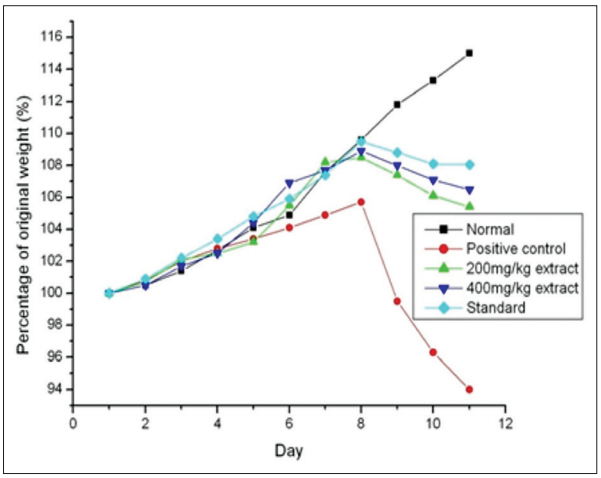

Figure 1: Effect of *Agave americana* Linn. leaf extract on body weight in indomethacin induced entero-colitis (body weight in percentage of original weight)

MPO and LPO activity

The MPO and LPO assay showed significant increase ($P < 0.001$) in MPO and LPO activity of positive control, whereas animals treated with extract and standard group have showed significant ($P < 0.001$) reduction compared to positive group. MPO and LPO activity of the drug-treated group was comparable with the standard group.

Table 2: Effect of *A. americana* Linn. leaf extract on ulcer score MPO activity and LPO assay in indomethacin induced entero-colitis

Groups	Ulcer score	MPO activity	LPO assay
Normal	0	1.345±0.1	0.264±0.02
Positive control	10.17±0.47***	8.44±0.21***	0.68±0.54***
200 mg/kg extract	3±0.2582###	4.36±0.1###	0.509±0.33##
400 mg/kg extract	2.33±0.21###	3.10±0.07###	0.471±0.01##
Standard	2±0.25###	2.06±0.08###	0.404±0.01###

***Significant increase in score values $P<0.001$ compared to normal group. ###Significant decrease in score values $P<0.001$ compared to positive control group. ##Significant decrease in score values $P<0.01$ compared to positive control group. *A. americana: Agave americana*, MPO: Myeloperoxidase, LPO: Lipidperoxidase

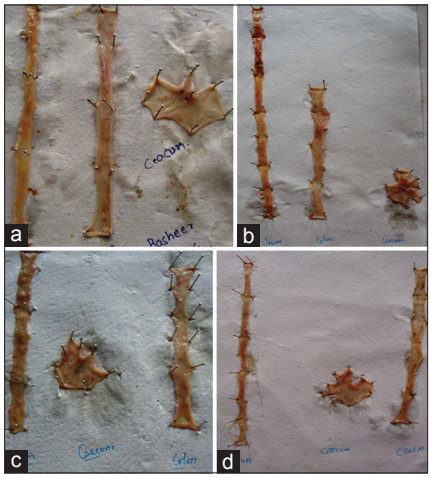

Figure 2: (a) Effect of *Agave americana* Linn. leaf extract on ulcer score in ileum, cecum and colon in normal control group, (b) effect of *A. americana* Linn. leaf extract on ulcer score in ileum, cecum, and colon in induced group, (c) effect of *A. americana* Linn. leaf extract on ulcer score in ileum, cecum, and colon in extract treated group, (d) effect of A. *americana* Linn. leaf extract on ulcer score in ileum, cecum, and colon in standard group

Histopathological examination of ileum and cecum

Histopathology of ileum [Figure 3] in normal rat (a) showing normal mucosa with intact epithelial surface, whereas indomethacin-induced group (b) showing massive necrotic destruction of epithelium. Treatment with extract of *A. americana* Linn. leaf 200 mg/kg and 400 mg/kg (c and d, respectively) showing decreased epithelial damage, regeneration, and suppressed inflammatory reaction. Standard group showed suppressed inflammatory reaction (e).

Histological sections of normal caecum [Figure 4] of rat (a) shows intact epithelium, indomethacin-induced enterocolitis (b) shows inflamed caecum with severe infiltration and edema. Treatment with extract 200 and 400 mg/kg (c and d respectively) showing submucosal edema and few inflammatory cells and standard treated rat showing little submucosal edema (e).

DISCUSSION

IBD refers to relapsing chronic inflammatory diseases of which CD and UC constitute two major forms.[1] The incidence of enterocolitis is approximately 10-20 per 10^5 per year in Western countries. The primary aims of medical therapy for patients with UC are directed at inducing and then maintaining remission of symptoms and mucosal inflammation to provide an improved quality of life with the least amount of steroid exposure.[23] According to traditional claims *A. americana* Linn. leaf exhibits anti-inflammatory wound healing and antioxidant property.[14,15] Decrease in body weight is one of the symptoms of enterocolitis. At the end of study body weight of induced rats was significantly decreased compared to normal group whereas extract and standard drug treated groups have retained significant body weight as shown in Table 1.

Mediators like TNF-α, interleukin 6 and LB_4 plays an important role in the inflammation of the intestinal mucosa in animal models of IBD. Results have shown significant decrease in ulcer score of extract treated animals compared to positive control group proving its beneficial effect.

In IBD, the inflammatory process is probably derived from the chronic presence of numerous activated MPO containing phagocytes in the inflamed intestine. MPO is an indicator

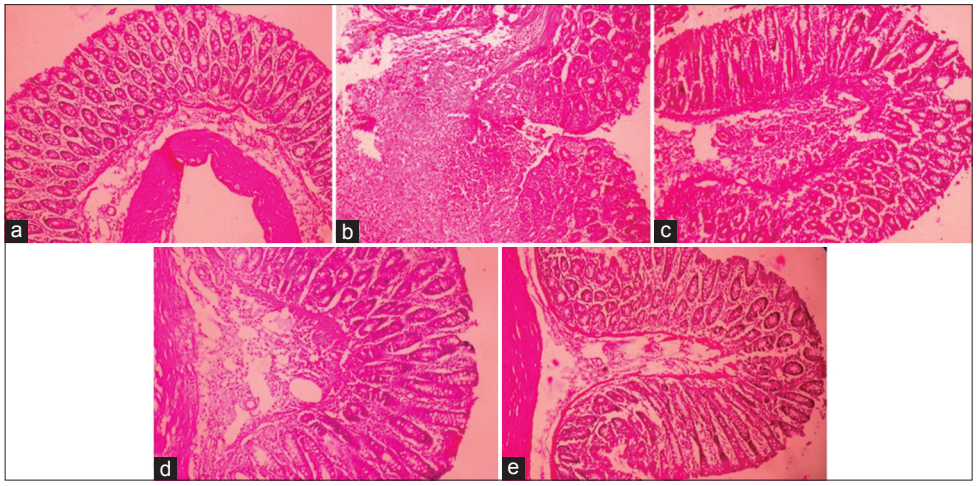

Figure 3: (a-e) Histopathology of ileum

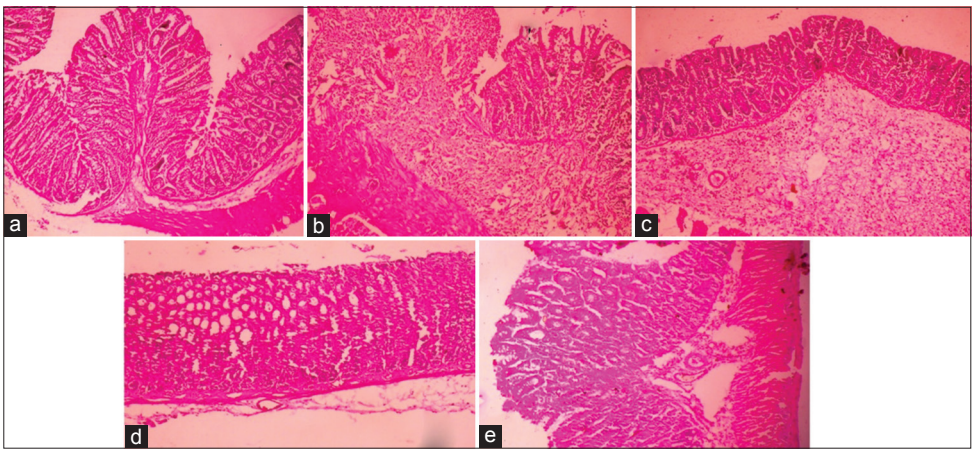

Figure 4: (a-e) Histopathology of cecum

of inflammation and mucosal damage. MPO assay has provided data supporting protective effect of extract showing a significant decrease in MPO activity compared to positive control.

Oxidative stress also plays an important role in the pathophysiology of IBD.[24] In patients with IBD the repeated cycle of injury of intestinal mucosa has been shown to increase the risk of colon cancer.[14] LPO assay is an indicator of oxidative stress and reactive metabolites. Results have shown there was significant increase in LPO activity in indomethacin group, whereas significant decrease in LPO activity has been observed in extract and standard drug treated groups.

Histopathological studies of ileum and cecum have shown sever necrosis and edema in induced group. Whereas extract treated groups have shown intact intestinal mucosa along with regeneration of intestinal epithelium cells providing protective mechanism. The major phytoconstituent present in *A. americana* are saponins. They are a vast group of glycosides, widely distributed in higher plants. Pharmacological properties of saponin include anti-inflammatory, antifungal, antibacterial, anti-parasitic, and antitumor activity.[25]

In all above studied, parameters extract treated groups have shown better results which are comparable with the standard prednisolone treated group. Possible mechanism of action may be by decreasing number of neutrophils and reduction in the synthesis of inflammatory mediators. *A. americana* is both anti-inflammatory and also has protective and regeneration property.

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