PROTECTIVE EFFECT OF ETHANOLIC ROOT EXTRACT OF *ARGYREIA SPECIOSA* AGAINST TRINITROBENZENE SULFONIC ACID INDUCED ULCERATIVE COLITIS IN RATS.

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Summary

Ulcerative colitis is a disease of immune disregulation that causes ulcers in the lining of the rectum and colon. The study of ethanolic root extract of *Argyreia speciosa* belonging to the family convolvulaceae was carried out to evaluate the effect in experimentally induced ulcerative colitis in rats. Adult Wistar rats of either sex were used (n = 36). Colitis was induced by a single intra-colonic application of 2,4,6-trinitrobenzene sulfonic acid(TNBS 20 mg) dissolved in 35% ethanol, into the descending colon. Rats were divided into six groups (n = 6). Group I received phosphate buffer saline, Group II TNBS, Group III, IV and V received TNBS + ethanolic root extract of *Argyreia speciosa* (with different doses of 50, 100 and 200 mg/kg), and Group VI received TNBS + Sulphasalazine (360 mg/kg). After completion of 14 days of treatment, animals were sacrificed and the following parameters were assessed: colon weight/length ratio, morphological score, histological examination, myeloperoxidase, malondialdehyde and superoxide dismutase activity. Anti-microbial activity of EREAS on standard microbial strains was studied by cup-plate and tube dilution method. TNBS induced ulcerative colitis caused increase in colon weight/length ratio, colonic tissue damage, myeloperoxidase activity, malondialdehyde activity and decrease in superoxide dismutase activity. Treatment with EREAS (100mg/kg, 200mg/kg) caused a dose dependent decrease in colon weight/length ratio, colonic tissue damage, myeloperoxidase activity, malondialdehyde activity and increase in superoxide dismutase activity. Our results indicate the efficacy of EREAS in TNBS induced experimental colitis model in rats. EREAS inhibited additional anti-microbial activity.

Keywords: Ulcerative colitis; *Argyreia speciosa*; 2,4,6-Trinitrobenzene sulfonic acid; Sulphasalazine; Myeloperoxidase; Malondialdehyde; Superoxide dismutase; Anti-microbial activity

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Introduction

Argyreia speciosa (Convolvulaceae), commonly known as elephant creeper is a woody climber distributed throughout India and has been used as a 'rasayana' drug in the traditional Ayurvedic system of medicine. The leaves are used by natives as a local stimulant and rubefacient in skin diseases, the seeds are a rich source of ergoline alkaloids while the roots are reported to be a tonic, aphrodisiac, bitter, diuretic and used in rheumatism, gonorrhea, chronic ulcer and diseases of nervous system[1,2]. Phytochemical screenings of the plant have shown the presence of alkaloids, flavonoids, triterpenes, phenylpropanoids, lipids, tannin and resin[3,4] . Pharmacological studies on Argyreia speciosa have been reported it to possess anti-inflammatory[5], anti-arthritic[5], immunomodulatory[6], wound healing[7], hepatoprotective activity[8] and nootropic effect [9].

Ulcerative colitis (UC) is a disease of immune disregulation that causes ulcers in the lining of the rectum and colon affecting the mucosa and sub mucosa of the colon and often remains limited to this level. In severe colitis, the tissue reaction extends through the muscle layer to the serosa, disrupting muscle and neural continuity, which results in loss of bowel tone[10,11].
An important marker of UC is mucosal neutrophil infiltration associated with mucosal damage. Neutrophil functions by phagocytosis mechanism and by production of reactive oxygen species, which plays an important role in host defense. Activated neutrophils are attracted to sites of inflammation, where they defend the host against invading microorganisms, this process causes tissue damage in certain inflammatory processes, including inflammatory bowel disease (IBD) [12].

Materials and methods

Plant material, chemicals, and drugs

Ethanolic root extract of Argyreia speciosa was procured from Green Chem. Pvt. Ltd, Bangalore, India (Batch No:ARG/8001). Sulfasalazine from Wallace pharmaceuticals, Mumbai, India, 2,4,6-trinitrobenzene sulfonic acid (TNBS) from Himedia Labs Pvt. Ltd. All other chemicals used were of analytical grade.

Animals

Adult Wistar rats of either sex weighing between (150–250g) were maintained under standard laboratory conditions maintained at 22 ± 2 ◦C, 12-h light–dark cycle, with free access to standard diet and water *ad libitum*. The approval of the Institutional Animal Ethical Committee (IAEC) of Al-Ameen College of Pharmacy Bangalore (Karnataka) was taken prior to the experiments and was conducted in strict compliance according to ethical principles and guidelines provided by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Induction of colitis

Colitis was induced by a single intra-colonic application of 20 mg TNBS dissolved in 35% ethanol using a rubber catheter of diameter 2 mm. The catheter was lubricated with glycerin and inserted rectally into the colon through anus such that the tip was 8cm inside from the anus. The total volume was expelled with additional air. Rats were observed for 2 weeks and sacrificed under anesthesia using anesthetic ether and by cervical dislocation for assessment of various parameters. During and after TNBS administration, the rats were kept in a head-down position until they recovered from the anesthesia for a few minutes to prevent leakage of the intracolonic instillation, and were returned to their cage.

Groups

The animals were divided into six groups, each containing six rats.

Group I animals were administered phosphate buffer saline intra colonically. Group II were administered TNBS (20mg) dissolved in 0.25ml of 35% ethanol. Group III, IV and V were administered TNBS (20mg) dissolved in 0.25ml of 35% ethanol + EREAS (50mg/kg, 100mg/kg and 200mg/kg, p.o). Group VI were administered TNBS (20mg) dissolved in 0.25ml of 35% ethanol + Sulphasalazine[13] (SSZ) 360 mg/kg, p.o. After 14 days of treatment, animals were sacrificed and the following parameters were assessed, colon weight/ length ratio, morphological index, histological index, myeloperoxidase activity, malondialdehyde activity, superoxide dismutase activity.

Assessment of colitis

The colonic segments were placed on an ice-cold plate, cleaned of fat and mesentery and blotted on filter paper. The colon was weighed/cm length and expressed as mg/cm tissue length[13].

Macroscopic assessment

The colon longitudinally opened and scored for macroscopically visible damage using magnifying lens according to the criteria, which take into account the extent as well as the severity of colonic damage[14].

Histopathological studies

The colonic specimens was taken from a region of the inflamed colon immediately adjacent to the gross macroscopic damage in the distal colon of each animal and was fixed in 4% buffered formaldehyde. Sections of tissue was cut (5µm), stained with hematoxylin and eosin (H and E) and evaluated by light microscopy for morphological changes[14]**.**

Biochemical parameters assessment

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte accumulation, was determined by the tetramethylbenzidine (TMB) method, Malondialdehyde (MDA) and superoxide dismutase (SOD) levels in the colonic tissue were determined[15,16].

Statistical analysis

All data are expressed as mean ± standard error of the mean (S.E.M.). Statistical analysis was performed using instat statistical software.

Analysis of variance (ANOVA) followed by Tukey Multiple Comparison Test. A value of $P< 0.05$ was considered as the level of significance.

Anti-microbial activity

Test microorganisms

Standard cultures *of Bacillus subtilis* (NCIM-2708)*, Staphylococcus aureus* (2079)*, Escherichia coli* (2685), *Staphylococcus epidermidis* (2478), *Aspergillus niger* and *Candida albicans* were obtained from Al-Ameen Biotechnology and Research center, Bangalore.

Preparation of test and standard solutions

The test solutions of extract were prepared in DMSO at a concentration of 0.5-1.5mg/ml. Ampicillin and Fluconazole was used as standard and was dissolved in sterilized water to get a concentration of 100 μg / 0.1 ml.

Screening for anti-microbial activity

Preliminary anti-bacterial and anti-fungal activity was determined, minimum inhibitory concentration; minimum bacterial concentration and minimum fungicidal concentration were calculated[17,18,19].

Results

Colonic mucosal damage

Rats administered TNBS (20mg single dose, intra colonically) showed significantly higher when compared to CW/LR value in normal control rats. TNBS treated rats administered EREAS showed CW/LR values significantly lower which was comparable to standard sulfasalazine treated group (Fig 1). The morphology of colon of TNBS treated rats' revealed inflammatory response with presence of inflammatory changes in the mucosa. EREAS and sulfasalazine group showed MI (macroscopic index) significantly lower compared to TNBS group.(Fig 2). However, the histopathology of colon administered with TNBS showed mucosal ulceration, transmural inflammation, diffuse infiltration of inflammatory cells in the mucosa and submucosa. The histopathology of colon administered with EREAS (50mg/kg, p.o) showed focal ulceration and inflammation extending to muscularis propria. The histopathology of colon administered with EREAS (100mg/kg and 200mg/kg p.o) showed healing of intestinal wall with inflammation limited only to submucosa and mucosa. The histopathology of colon administered with standard sulfasalazine showed healing of intestinal wall with reduced inflammation limited to mucosa (Fig 3) which was taken as positive contol.

Myeloperoxidase activity, lipid peroxidation and anti-oxidant activity

The tissue Myeloperoxidase (MPO) and Malondialdehyde (MDA) levels marker for lipid peroxidation in TNBS administered group showed significantly higher when compared to MPO and MDA levels in normal control rats. The tissue MPO and MDA levels in rats administered with EREAS (100mg & 200mg/kg p.o) showed significantly lower when compared to TNBS administered rats. The tissue MPO and MDA levels in rats administered with sulfasalazine showed significantly lower when compared to TNBS administered group (Fig 4, 5). The tissue superoxide dismutase (SOD) level in TNBS administered group showed significantly low when compared to superoxide dismutase in normal control rats.

The tissue SOD level in rats administered with EREAS (100mg & 200mg per kg p.o) showed significantly higher when compared to TNBS administered rats. The tissue SOD levels in rats administered with sulfasalazine showed significantly higher when compared to TNBS administered rats (Fig 6).

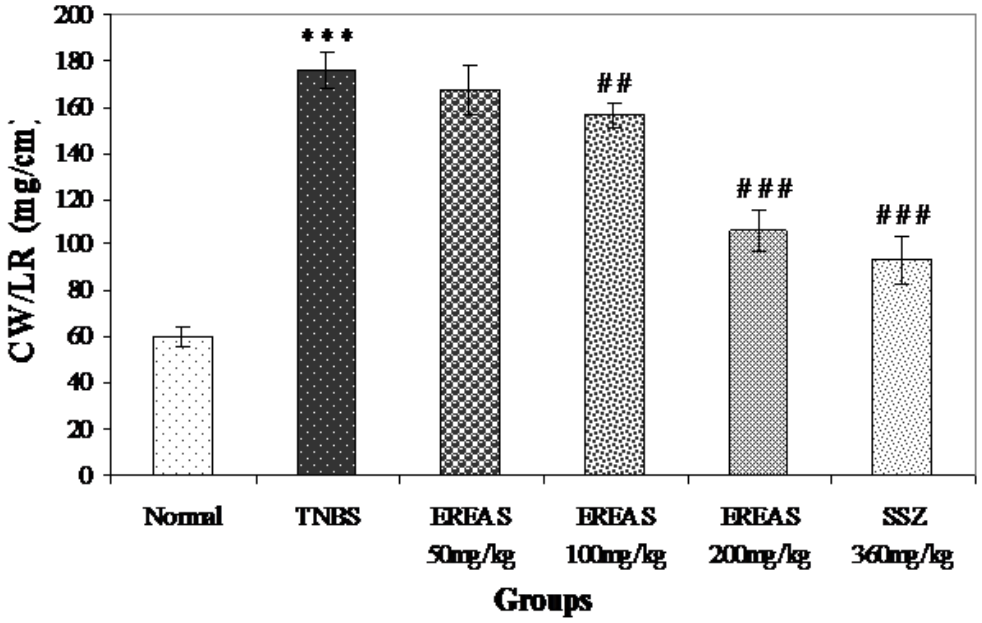

Figure: 1 Effect **of EREAS treatment on colon weight/length ratio of colon in TNBS induced ulcerative colitis in rats.**

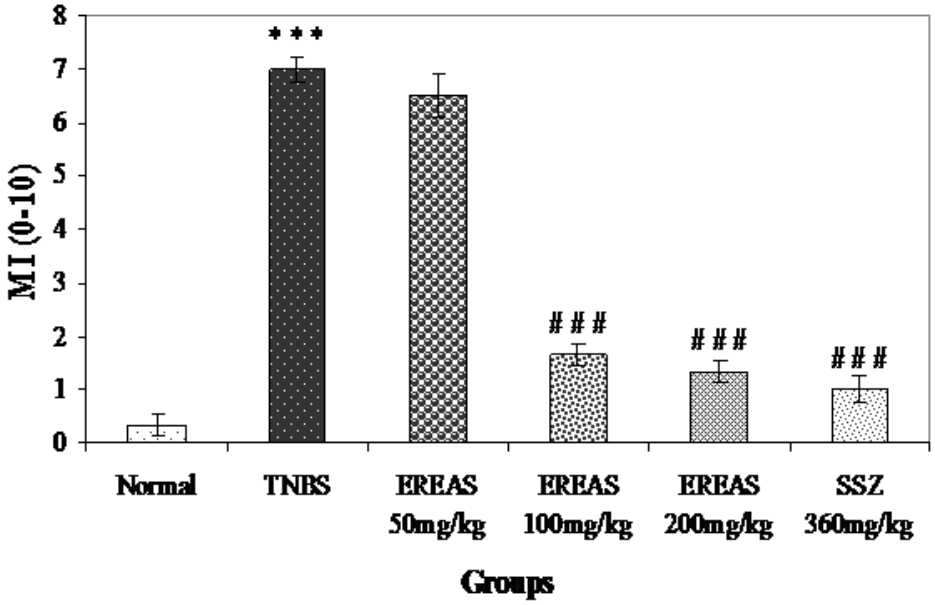

Figure: 2 Effect of EREAS treatment on macroscopic index of colon in TNBS induced ulcerative colitis in rats.

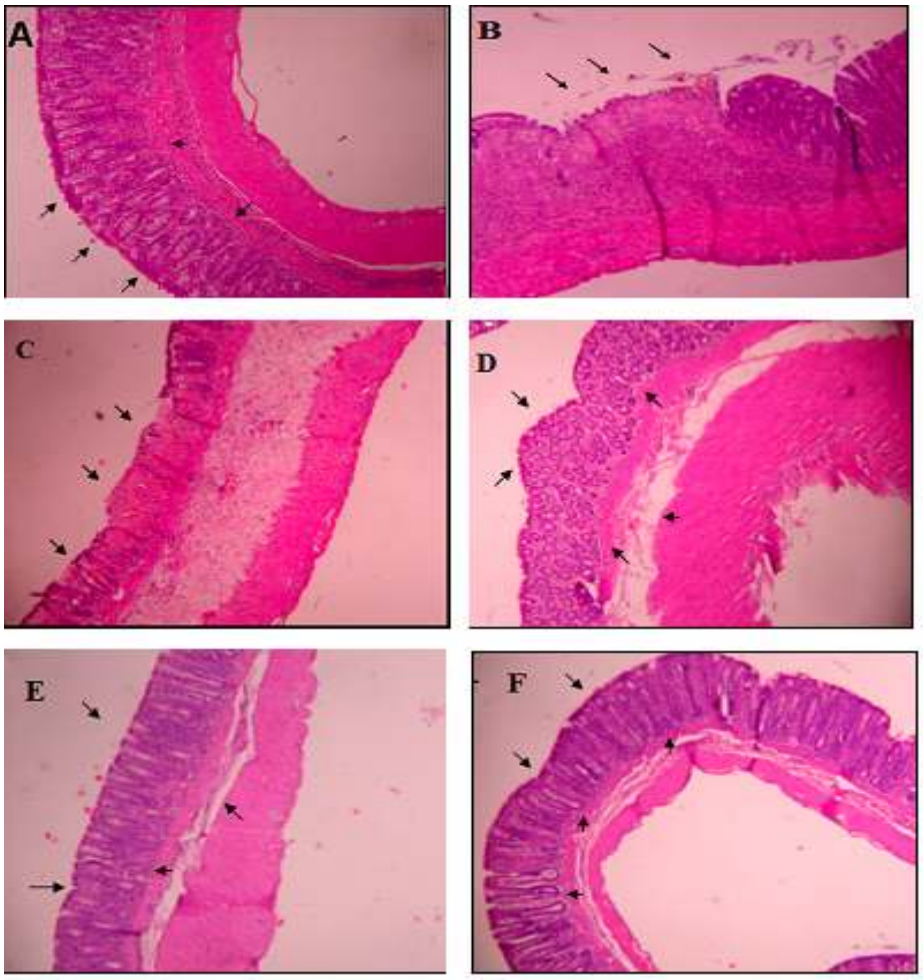

Figure: 3 (A) Histological sections of H & E stained colonic mucosa of rat in normal group showing normal histology of rat colon. (B) Colonic mucosa of rat administered TNBS showing mucosal ulceration and transmural inflammation most evident in the enlarged sub mucosa of rat colon. (C) Colonic mucosa of rat administered EREAS 50mg/kg showing focal ulceration and inflammation with involvement of muscularis propria. (D) Colonic mucosa of rat administered EREAS 100mg/kg showing healing intestinal wall with inflammation limited to mucosa and submucosa. (E) Colonic mucosa of rat administered EREAS 200mg/kg showing healing intestinal wall with inflammation limited to mucosa. (F) Colonic mucosa of rat administered SSZ 360mg/kg showing healing intestinal wall with reduced inflammation limited to mucosa

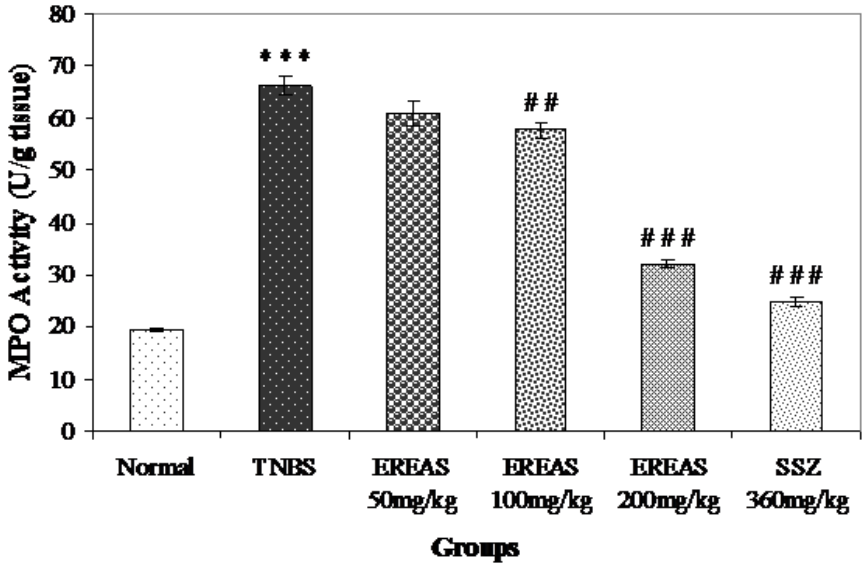

Figure: 4 Effect of EREAS treatment on MPO activity of colon in TNBS induced ulcerative colitis in rats.

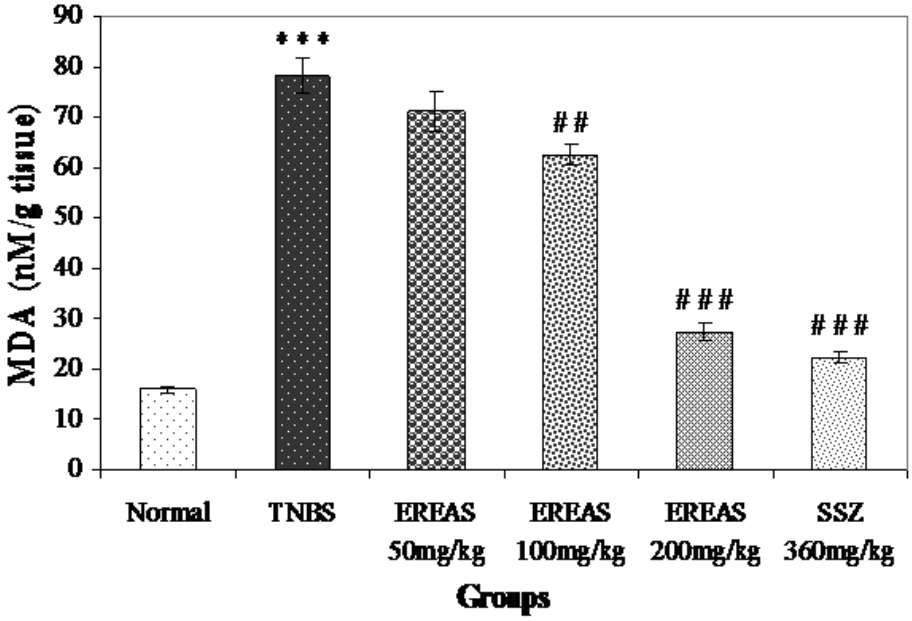

Figure: 5 Effect of EREAS treatment on MDA of colon in TNBS induced ulcerative colitis in rats.

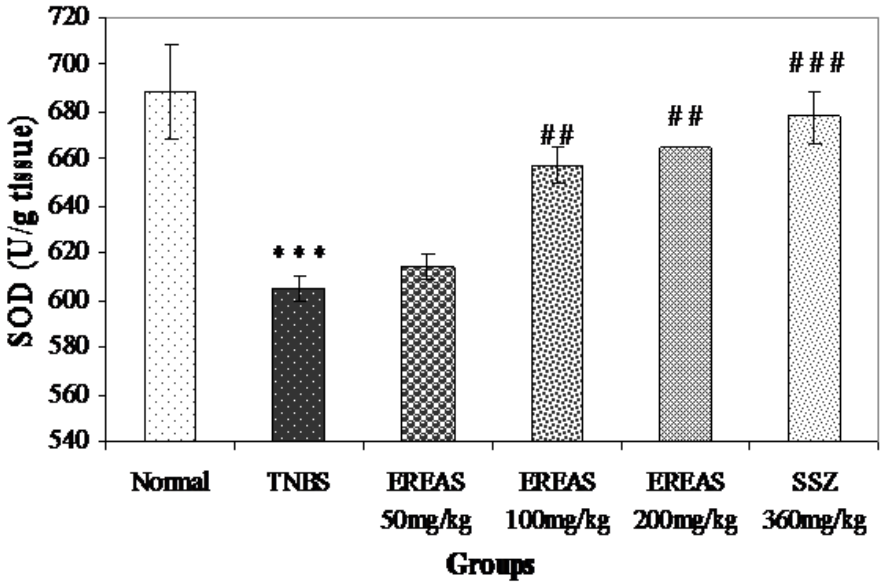

Figure: 6 Effect of EREAS treatment on SOD of colon in TNBS induced ulcerative colitis in rats.

Score data are expressed as mean ± S.E.M, n=6

Comparison of TNBS with normal group ***(P<0.001)

Comparison of EREAS groups and SSZ group with TNBS group $^{\#\#}$ (P<0.01), $^{\#\#\#}$(P<0.001)

Anti-microbial activity of EREAS

EREAS gave favorable results against all the tested micro-organisms with zone of inhibition between 7-20mm. the present study reveals that EREAS was effective against S. aureus, B. subtilis, S. epidermidis, E. coli, C. albicans and A. niger in dose dependent manner. The higher zone of inhibition was recorded at 1.5mg/ml concentration than 0.5mg/ml concentration of EREAS. As the dosage level increases, the inhibitory effect also increased. The EREAS was screened for its antibacterial property against human pathogenic Gram positive, Gram negative bacteria and against fungi. Ampicillin and fluconazole were used as the standard respectively.

EREAS was found to be active at the concentration of 0.5-1.5mg/ml against the selected strains of organism. Hence to further confirm the exact concentration of their antibacterial activity, MIC determination was carried out using broth dilution method. In broth dilution method the inhibition in growth at different concentration is measured by comparing the turbidity of the medium with the control having no organism, to confirm the MIC, the medium in the tube was streaked on the sterile nutrient agar media and observed next day for growth. The Petri dish showing the growth of few colonies is considered the MIC and if the next higher dilution is not showing any growth it was considered as minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC).

The results in terms of µg/ml for extract against different organism are reported in Table No, 4. The EREAS exhibit good activity with MIC values ranging from 400-900µg/ml, MBC and MFC values ranging from 500-700 and 1000 µg/ml.

		Zone of inhibition (mm)				
Sl.No	Organisms	EREAS 0.5mg/ml	EREAS 1mg/ml	EREAS 1.5mg/ml	Ampicillin 100µg/ml	Fluconazole 100µg/ml
1	*S.a*	10	14	20	23	-
2	*B. s*	8	17	18	24	-
3	*S.e*	11	15	18	23	-
4	*E.coli*	8	18	19	23	-
5	*C.a*	7	14	16	-	21
6	*A.n*	7	12	13	-	20

Table: 1. Preliminary antibacterial and antifungal activity of EREAS

S.a-Staphyllococcus aureus, B.s-Bacillus subtilis, S.a-Staphyllococcus epidermidis, E.coli-Escherichia coli, C.a-Candida albicans, A.n-Aspergillus niger

		Concentration in µg/ml of EREAS		
Sl.No	Organisms	MIC	MBC	MFC
1	*S.aureus*	500	700	-
2	*B. subtilis*	400	600	-
3	*S.epidermititis*	400	500	-
4	*E.coli*	500	700	-
5	*C.albicans*	800	-	1000
6	*A.niger*	900	-	1000

Table: 2. Minimum inhibitory concentration, minimum bactericidal concentration, minimum fungicidal concentration of EREAS.

Discussion

Induction of inflammation in animal model of colitis is associated with derangement of epithelial barrier and transport function. The early phase of these changes was produced by ethanol which is used in this experimental model precisely because it is a barrier breaker. After destruction of mucosal integrity by ethanol, TNBS which is a hapten, binds to a high molecular tissue protein and turns into an antigen, which is recognized by macrophages and presented to sensitize T-lymphocytes[18,19]. Administration of TNBS with ethanol to rats promotes an experimental ulcerative colitis which possesses certain pathological features of

ulcerative colitis seen in humans. These features include extensive ulceration of epithelial layer, massive bowel wall edema, fibrotic thickening of mucosa and dense cellular infiltrate characterized by neutrophils.

In present study there was an increase in colon weight /length ratio, extensive colonic mucosal and submucosal damage characterized by infiltration of inflammatory cell and ulcer formation after administration of TNBS. Morphological and histological score of colonic tissue in TNBS treated rats administered EREAS (100mg/kg and 200mg/kg, p.o) was reduced compared to rats administered TNBS alone. Thus EREAS acts through the effect on polymorphonuclear cell functioning; it reduces phagocytosis by inhibiting polymorphonuclear leucocytes.

Myeloperoxidase is an enzyme found in neutrophils and it is used as quantitative index of inflammatory damage[19]. Myeloperoxidase is secreted by the neutrophils whenever there is inflammation and therefore the number of neutrophils is directly co-related with myeloperoxidase activity. Neutrophils play an important role in producing superoxide anion and a cascade of various reactive species leading to a very reactive hydroxyl and peroxide radicals[20]. Reduction in the activity of myeloperoxidase enzyme can be interpreted as a manifestation of the anti-inflammatory activity of a drug[21]. In present study there was an increase in MPO after administration of TNBS (single dose of 20mg dissolved in 35% ethanol intra colonically). However, colons from rats administered with EREAS (100mg/kg and 200mg/kg, p.o) showed reduction in the activity of MPO. The reduction in the MPO activity was confirmed histologically, since the level of leukocyte infiltration in the colonic mucosa was lower in EREAS (100mg/kg and 200mg/kg, p.o) administered rats than in the corresponding from rats administered TNBS significantly lower than those from rats administered TNBS alone. This may be due to anti-inflammatory activity of EREAS [6].

Phytochemical investigations on *Argyreia speciosa* have shown the presence of alkaloids, flavonoids, triterpines and phenyl-propanoids[3,4]. The anti-inflammatory activities, hepatoprotective activity of EREAS have been reported to be due to the presence of flavonoids [5,7].

Infiltration of leukocytes into the mucosa has been suggested to contribute significantly to the tissue necrosis and mucosal dysfunction associated with colitis as they represent a major source of reactive O_2 radicals in the inflamed mucosa. These reactive oxygen species degrade polyunsaturated lipids, forming malondialdehyde. This compound is a reactive aldehyde and is one of the many reactive electrophile species that cause toxic stress in cells and form advanced glycation end products. The production of this aldehyde is used as a biomarker to measure the level of oxidative stress[15,16]. Thus, increased colonic tissue level of MDA is used as one of the parameters to study the tissue damage via lipid peroxidation. This inhibition of the generation of malondialdehyde and lipid peroxidation may possibly help to decrease the tissue damage. In present study there was an increase in MDA level after administration of TNBS (single dose of 20mg dissolved in 35% ethanol intra colonically). However, colons from rats administered with EREAS (100mg/kg and 200mg/kg, p.o) showed MDA levels significantly lower than those from rats administered TNBS alone, which could be due to inhibition of lipid peroxidation by EREAS.

The role of superoxide dismutase (SOD) is highly significant in maintaining cell integrity. The principle free radical in tissues is superoxide anion (O_2^-) which is converted to secondary oxidant H_2O_2 by SOD. The oxygen free radical (O_2^-) is also produced by both endothelial cells through xanthine oxidase and activated neutrophil through NADPH oxidase, which reduces the molecular oxygen to (O_2^-), and through the enzyme MPO. This enzyme catalyses the formation of such potent cytotoxic oxidants as hypochlorous acid from H_2O_2 and chloride[15,16]. In present study, there was decrease in SOD level after administration of TNBS. However, colons from rats administered with EREAS (100mg/kg and 200mg/kg, p.o) showed SOD levels significantly high than those from rats administered TNBS, which is due to anti-oxidant property of EREAS. Thus, in conclusion present study reveals that EREAS possesses dose dependent anti-inflammatory and anti-oxidant properties comparable to sulfasalazine effects. This effect is due to its anti-inflammatory, anti-oxidant activity and inhibition of lipid peroxidation.

Beside the anti-inflammatory and anti-oxidant effect of EREAS, it shows the anti-microbial activity.

Conclusion

It may be concluded that ethanolic root extract of *Argyreia speciosa* exerts a significant protection against trinitrobenzene sulfonic acid induced ulcerative colitis in rats. EREAS possesses dose dependent anti-inflammatory and anti-oxidant properties comparable to standard sulfasalazine effects. This effect is due to its anti-inflammatory, anti-oxidant activity and inhibition of lipid peroxidation. Beside the anti-inflammatory and anti-oxidant effect of EREAS, its anti-microbial activity may add to its beneficial effect against ulcerative colitis disorder.

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