RESEARCH ARTICLE

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Histopathological Evaluation of *Tridax procumbens* Linn Potential in Ulcerative Colitis

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Abstract: *Tridax procumbens* Linn. (Family: Asteraceae) is being used in traditional medicine for the prevention and treatment of ulcer. So the aim of study was to evaluate antiulcer activity of petroleum ether and methanol extracts of *Tridax procumbens* Linn to validate its traditional use. In the present study petroleum ether, methanol extract of *Tridax procumbens* Linn at the doses of 100 mg/kg was evaluated for antiulcer activity using *in-vivo* acetic acid induced ulcerative colitis in mice. The results of present investigation showed that the methanol extract of *Tridax procumbens* Linn at (100 mg/kg, i.p.) significantly shows ulcer index 1.23 and percent ulcer protection 57.12 as compared to standard drug prednisolone with ulcer index 1.51 and percent ulcer protection 50.60. From the above study it can be concluded that the methanolic extract of whole plant of *Tridax procumbens* Linn shows ulcer prevention and protection activity can be used for prevention and treatment of ulcerative colitis.

INTRODUCTION

Inflammatory bowel disease encompasses two idiopathic, chronic, inflammatory diseases: Crohn's disease and ulcerative colitis. Crohn's disease and ulcerative colitis are disorders of unknown cause involving genetic and immunological influence on the gastrointestinal tract's ability to distinguish foreign from self-antigens. They share many overlapping epidemiological, clinical and therapeutic characteristics. In some patients, it is not possible to distinguish which form of inflammatory bowel disease is present. There are, however, important pathological and clinical differences that distinguish these inflammatory disease processes. Clinically, Crohn's disease tends to present more frequently with abdominal pain and perianal disease, whereas ulcerative colitis is more often characterized by gastrointestinal bleeding. Cobblestoning mucosa and aphthous or linear ulcers characterize the endoscopic appearance of Crohn's disease. Ulcerative colitis presents with diffuse continuous involvement of the mucosa. Radiographic studies of patients with Crohn's disease characteristically show fistulas, asymmetry and ileal involvement. In contrast, radiographic studies of patients with ulcerative colitis show (Figure 1) continuous disease without fistulizing or ileal disease. [1]

Tridax procumbens L. is a common medicinal herb used by ethno-medical practitioners, belonging to family Asteraceae. It is best known as a widespread weed and pest plant. It is native to tropical America but it has been introduced to tropical, subtropical and mid temperate regions worldwide. The plant is a procumbent herb and is valued for its pharmaceutical properties. [2, 3]

Tridax procumbens L. is commonly known as 'Ghamra' and in English popularly known as 'coat buttons' because of the appearance of its flowers. Tridax plant is present throughout India and is employed as indigenous medicine for variety of ailments. It has been extensively used in Ayurvedic system of medicine and is dispensed as "Bhringraj" by some practitioner of Ayurveda which is well known medicine for liver disorder. It has been found to possess significant medicinal properties against blood pressure, bronchial catarrh, malaria, dysentery, diarrhea, stomach ache, headache, wound healing, it also prevents hair fall and check hemorrhage from cuts and bruises. [4] Its flowers and leaves possess antiseptic, insecticidal and parasiticidal properties. [3, 5] The plant also shows various pharmacological activities like Immunomodulatory, antidiabetic, anti hepatotoxic and anti-oxidant, anti-inflammatory, analgesic and marked depressant action on respiration. [5-11]

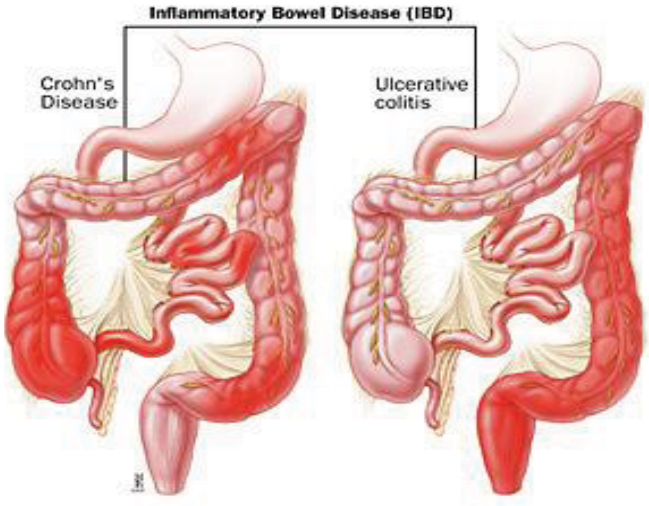

Figure 1: Anatomic distribution of crohn's disease and ulcerative colitis

MATERIALS AND METHODS

Experimental Animals

Animal models chosen for study are male albino mice (Swiss strain) weighing 22-25 g and male albino rats of wistar strain weighing 120-180 g. The Institutional Animals Ethics Committee approved the protocol vid no IAEC/NIB/19/2014.

1. Species: Mice and rats.
2. Strain: Albino.
3. Sex: Male.
4. Body weight: 22-25 g.
5. Housing and keeping They were housed in groups of six under standard laboratory conditions of temperature (25±2°C) and 12/12 hr light/dark cycle.
6. Diet and Water Animals had free access to standard pellet diet and water *ad libitum*.

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Table 1: Treatment Summary

Group	Treatment
Control	0.1 ml of 6% acetic acid once intrarectally.
Test	7 day pretreatment with extract + on 8th day 0.1 ml of 6% acetic acid once intrarectally 3 cm from the anal margin. Drug treatment continued up to 10th day.
Prednisolone treatment	Started on day of acetic acid treatment, given orally as a suspension containing 0.5% sodium CMC. Dose - 1.14 mg/Kg for 3 days. + On 8th day 0.1 ml of 6% acetic acid once intrarectally.

Preparation of Standard Drug and Extract Solution
Solution of all extracts was prepared in 5 % Polyethylene glycol. Test drug was dissolved in distilled water or in physiological salt solution. Prednisolone was dissolved in physiological saline.

Plant Material
The plant of *Tridax procumbens* L. (Asteraceae) were identified and located as a tree near Anjneri hills at Trimbakeshwar, District Nashik, MS, India, with the help of botanist and drugs identity was confirmed by comparing the samples with the description mentioned in the different floras and text. Further to authenticate the plant species herbarium sheet was prepared and forwarded to BSI (Botanical Survey of India, Pune). A voucher specimen (PAR 01) deposited at BSI. Whole plant was collected in the month of September cleaned thoroughly, dried at 30-40°C in oven and coarse powder of it was stored at 25°C in air tight container.

Drugs and Chemicals
Prednisolone and Phenobarbital were purchased from Medico remedies, Mumbai, India and Human Antibiotic Pharmaceuticals (Brand:Barbinaol 100) respectively. While sodiun Petroleum ether, Methanol, acetic acid, sodium CMC, formaline, xyline, alcohol, paraffin wax, albumin, ammonia , eosin were purchased from Himedia, Mumbai, India.

Storage of Drug Solution
Fresh drug solutions were prepared for each day's work. The solutions was kept in air-tight amber colored bottles and stored at room temperature till use.

Volume of the Injected Drug Solution
The volume of drug solution was calculated based upon the body weight of the animal.

Routes of Administration
All drugs were administered as per screening model procedures.

Statistical Analysis
All values are expressed as mean±SEM. Statistical significance was calculated using one-way ANOVA followed by Dunnet test.

Acute Toxicity Study (LD_{50} Determination) of Different Crude Extracts
Before exploring any new drug moiety, be a natural or synthetic, its safety studies have to be performed in order to find out the therapeutic window, minimum effective concentration and toxic dose level. This is done to assess that till which concentration, the drug under investigation is safe to be further explored for its therapeutic usefulness. Previously, the Lethal Dose studies used to be performed, known as LD_{50} determinations, in which the dose at which 50% of the animals die, was calculated to estimate the toxicity level of the drug and was a determinant factor for calculation of the therapeutic dose. The acute oral toxicity study of different crude extracts of selected plant material was carried out as per the guidelines set by Organization for Economic Co–operation and Development (OECD) guideline 423. OECD Guidelines for the Testing of Chemicals are periodically reviewed in the light of scientific progress or changing assessment practices. The original Guideline 423 was adopted in March 1996 as the second alternative to the conventional acute toxicity test, described in Test Guideline 401.

Principle of the Test [12]
It is the principle of the test that based on a stepwise procedure with the use of a minimum number of animals per step; sufficient information is obtained on the acute toxicity of the test substance to enable its classification. The substance is administered orally to a group of experimental animals at one of the defined doses. The substance is tested using a stepwise procedure, each step using three animals of a single sex (normally females). Absence or presence of compound-related mortality of the animals dosed at one step will determine the next step, i.e.:

1. No further testing is needed.
2. Dosing of three additional animals, with the same dose.
3. Dosing of three additional animals at the next higher or the next lower dose level.

***In-vivo* Assessment of Acetic Acid Induced Ulcerative Colitis in Mice**

a. Ulcer Index
b. Histopathology

1. Treatment Schedule

a. Group I: Prednisolone Standard (5 mg/kg) p. o.
b. Group II: Petroleum ether (100 mg/kg) p. o.
c. Group III: Methanol (100 mg/kg) p. o.
d. Group IV: Negative Control (Distilled water containing 20% Tween 80) p. o.

2. Procedure

a. Study comprises of four different groups (n = 6) as summarized in treatment schedule.

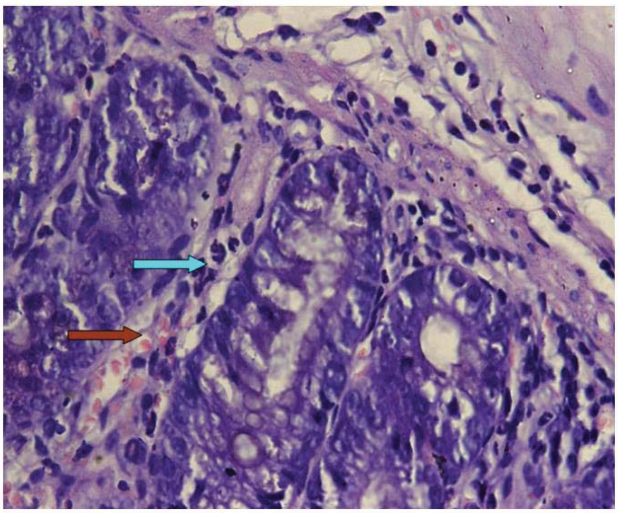
Table 2: Acute Toxicity Test (LD50 determination) of Different Crude Extracts

S. No.	Plant Material	Extracts	LD_{50} Cut-off mg/kg b. w.
1	Powder of *Tridax Procumbens* L.	Petroleum ether (60-80°C)	2000 mg/kg
		Methanol	2000 mg/kg

Table 3: Histopathological Observation

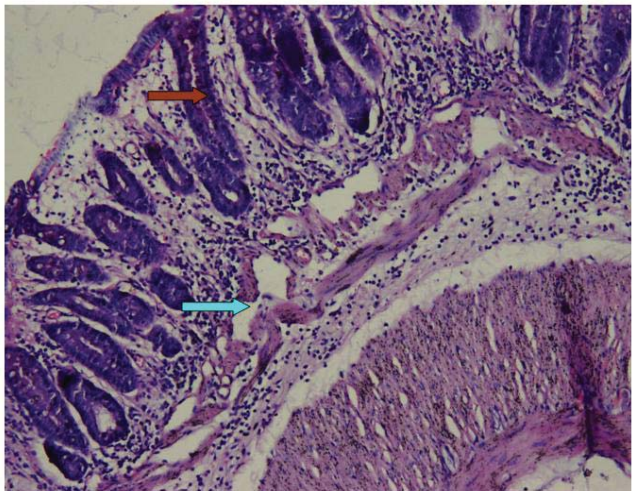
Treatment Group	Ulceration	Hyperemia	Necrosis	Edema	Cellular in Filtration	Goblet Cell Hyperplasia
Standard Prednisolone (5 mg/kg)	++	++	++	++	++	++
Pet. Ether extract 100 mg/kg	+++	+++	++	++	+++	+++
Methanol extract 100 mg/kg	++	++	++	++	++	++
Control, Acetic acid (negative)	++++	++++	++++	++	+++	+++

+: damage/ active changes up to less than 25 %, ++: damage/ active changes up to less than 50 %, +++: damage/ active changes up to less 75 %, ++++: damage/ active changes up to more than 75 %



Red arrow - Hemorrhages, Blue arrow - Cellular infiltration

Figure 2: Showing prednisolone treated group



Red arrow - Hemorrhages and ulceration, Blue arrow - Cellular infiltration and edema

Figure 3: Showing petroleum ether extract treated group

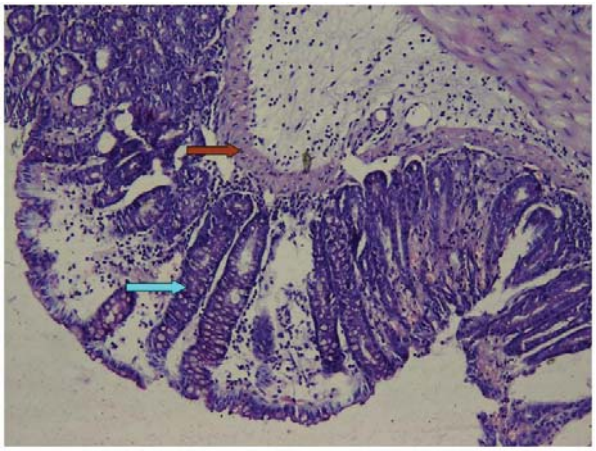
b. Test animals in group II to III receives seven day treatment of the different crude extract as mentioned in treatment schedule.
c. On eighth day all animals receives 0.1 ml 6% acetic acid intrarectally.
d. Prednisolone treatment in standard group was started on the day of acetic acid treatment.
e. Drug treatment in all groups was continued up to 10th day.
f. Control group receives only 0.1 ml 6% acetic acid intrarectally on eighth day.
g. After 48 hours of colitis induction mice were sacrificed by cervical dislocation and dissected upon to remove colon.
h. Five centimeters long piece of colon was flushed gently with saline, cut upon and scored for inflammation based on the macroscopic features.
i. Tissues were fixed in 10% formalin saline and examined histopathologically.

3. Induction of Colitis in Mice

Overnight fasted mice, anaesthetized by pentobarbital sodium (55.00 mg/Kg i.p.) 0.1 ml of 6% acetic acid once intrarectally. Allow to hang in air by holding tails for 1 – 2 min.

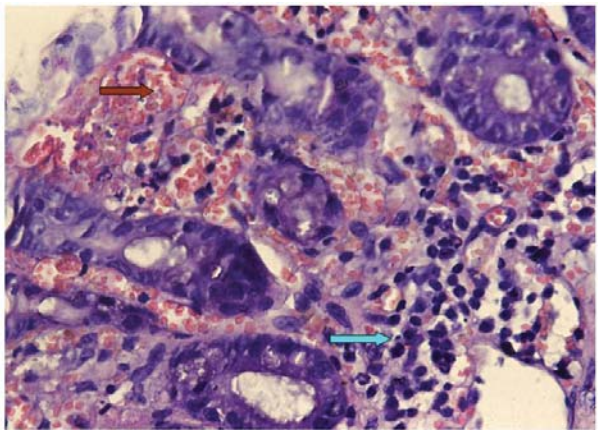
4. Methodology for H and E Staining Procedure

a. Fixation of the tissues: formalin 10 % was used for the fixation of the tissues. Tissues were fixed for the period of 24-36 hrs in 10 % formalin.
b. Tissues were trimmed at suitable size.
c. Washed under running tap water for 2 hrs.
d. Then tissues were dehydrated with the help of increasing grades of the alcohol.
e. 50% alcohol overnight.
f. 70% alcohol 2 hrs.
g. 80% alcohol 2 hrs.
h. 90% alcohol 2 hrs.
i. Absolute alcohol 2 hrs.



Red arrow - Hemorrhages, Blue arrow - Leucocytic infiltration

Figure 4: Showing methanol extract treated group



Red arrow -hemorrhages and ulceration, Blue arrow - Leucocytic infiltration

Figure 5: Showing negative treated group

j. Cleared with xyline 1 hrs (three changes each).
k. Embedded with paraffin wax at 60°C.
l. Blocks were prepared and blocks were stored at freezer for 4-5 hrs.
m. Slices of tissues were cut at 5 micron meter thickness
n. Cut slices were taken on the clean grease free glass slides smeared with egg albumin in water bath at temperature 60°C.

5. Staining Procedure

a. Deparafinnazation partially with heat and followed by immersing in the xyline for 3 min in each (three changes of three minutes each).
b. Sections rehydrated with decreasing grades of the alcohol.
c. 100% - 90% - 80% - 50% (3 min in each).
d. Slides were kept in distilled water for 5 minutes.
e. Kept in hematoxyline for 10 minutes.
f. Washed under running tap water for the period of 10 minutes.
g. One deep was given in 1 % ammonia water and immediately washed under tap water for 5 minutes.
h. 2-3 dips were given in alcoholic Eosin and slides again dehydrated with increasing grades of alcohol = 70, 80, 90 and 100 % alcohol (three minutes in each alcohol).
i. Slides were clear with xyline for 3 min in each jar (three changes).
j. Slides were mounted with DPX mountant.
k. And observed under suitable magnification.

6. Ulcer Index

Ulcer index is calculated by using following formula:

$$\frac{\text{Grade of ulcer in positive control} - \text{grades of ulcers in test}}{\text{Grades of ulcer in test} - \text{grades of ulcer in normal control}} \times 10$$

RESULTS

Acute Toxicity Studies

According to OECD guideline and acute toxicity studies, LD_{50} cut off dose for Petroleum ether and methanol extract was found to be 2000 mg/kg (Table 2).

Histopathological Study

Histopathological observations shown in Table 3. The treatment group I- standard prednisolone (5 mg/kg) shows (Figure 2) damage up to less than 50%. The treatment group II petroleum ether extract (100 mg/kg) shows (Figure 3) damage up to less than 75%. The next treatment group III Methanol extract (100 mg/kg) shows (Figure 4) damage up to less than 50%. The last treatment group IV control acetic acid (negative) shows (Figure 5) damage up to more than 75%.

Ulcer Index

As per the observation shown in Table 4 and Figure 6. The treatment group I prednisolone shows the ulcer index was 1.51 and percent ulcer protection 50.60%. The ulcer index of treatment group II petroleum ether extract (100 mg/kg) was 2.45 and percent ulcer protection 21.61%, while methanol extract (100 mg/kg) shows ulcer index 1.23 and percent ulcer protection 57.12%. The ulcer index for acetic acid (negative) was 3.62.

DISSCUSION

Acute Toxicity Studies

Animals were observed initially after dosing at least once during the first 30 minutes, periodically during the first 24 hours. In all cases death was observed within first 24 hours. Additional observations like changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and motor activity and behaviour pattern. Attention was also given to observation of tremors and convulsions. According to acute toxicity studies LD_{50} cut off dose for Petroleum ether and methanol extract was found to be 2000 mg/kg (Table 2).

Histopathological Study

The test procedure contains 0.1 ml of 6% acetic acid once intrarectally. Then 7 day pretreatment with extract and on 8^{th} day 0.1 ml of 6% acetic acid once intrarectally 3 cm from the anal margin. Then drug treatmant continued up to 10^{th} day (Table 1). After 48 hrs. of colitis induction mice were sacrificed by cervical dislocation and dissected upon

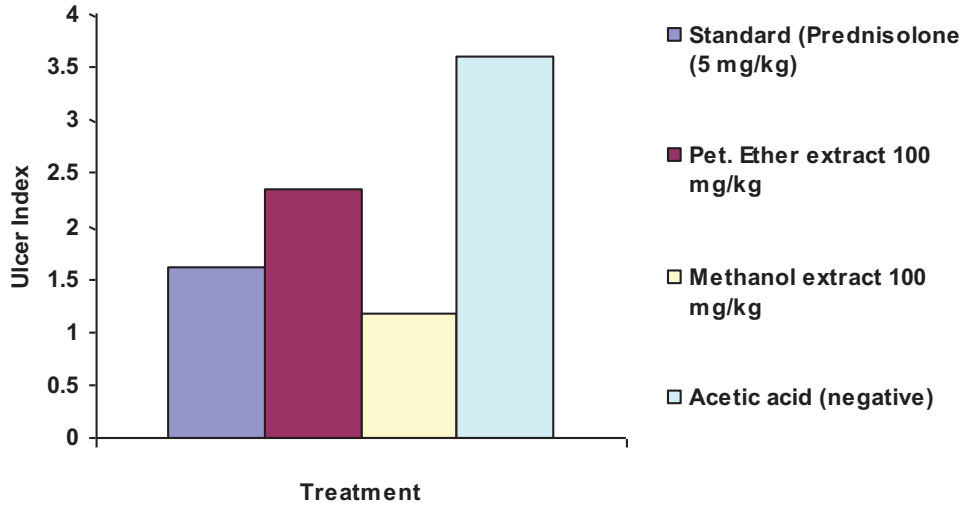


Figure 6: Ulcer index of various extracts

Table 4: Determination of Ulcer Index

Treatment Group	Ulcer Index	Percent Ulcer Protection
Prednisolone (5 mg/kg)	1.51	50.60
Pet. Ether extract 100 mg/kg	2.45	21.61
Methanol extract 100 mg/kg	1.23	57.12
Acetic acid (negative)	3.62	00.00

to remove colon. Five centimeters long piece of colon was flushed gently with saline, cut upon and scored for inflammation based on the macroscopic features. Tissues were fixed in 10% formalin saline and examined histopathologically.

As per the observations in Table 3 methanol extract (100 mg/kg) shows damage to epithelial cell of colon up to or less than 50% as same as standard drug Prednsolone (5 mg/kg) which also shows damage up to less than 50%. While Petroleum Ether extract (100 mg/kg) shows damage to epithelial cell of colon up to or more than 75% same as acetic acid negative control group. Hence methanol extract of whole plant of *Tridax procumbens* Linn may useful in prevention and treatmnet of ulcerative colitis.

Ulcer Index

Percent ulcer protection of Methanol extract of whole plant of *Tridax Procumbens* Linn (100 mg/kg) was found to be 57.12% which was greater than prednisolone (50.60 %) and Petroleum ether extarct (100 mg/kg) which was 21.61%. (Table 4 and Figure 6) hence, methanol extract of whole plant of *Tridax procumbens* Linn may useful in prevention and treatment of ulcerative colitis.

CONCLUSION

In present study our aim was to validate folk and ethnobotanical claim of *Tridax Procumbens* in prevention and treatment of ulcerative colitis. from the above study it can be concluded that the methanolic extract (100 mg/kg) of whole plant of *Tridax procumbens* Linn shows ulcer prevention and protection activity and may be useful for prevention of ulcerative colitis.

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